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13. ABSTRACT *(Maximum 200 words)*

The goal of this research project was to study the requirements for T cell activation in generating a potent anti-tumor immune response. In the first year, using a transplantable mammary carcinoma model, we demonstrated that the combination of CTLA-4 blockade and a GM-CSF-expressing vaccine was effective for treatment of recently established tumors. This funded year, these findings were to be expanded to 2 additional models: a transplantable melanoma model and a primary prostate cancer model, resulting in one manuscript recently published (1), and one submitted (2). In the melanoma model, we demonstrated that CTLA-4 blockade, in combination with a GM-CSF-expressing vaccine, could eradicate both subcutaneous tumors as well as lung metastases. More strikingly, mice that rejected tumors underwent a progressive depigmentation, reminiscent of the vitiligo that occurs in melanoma patients undergoing immunotherapy (3). In the transgenic prostate cancer model, we demonstrated that primary, autochthonous prostate tumor incidence is reduced and the histologic severity of tumor lesions is reduced in mice treated with anti-CTLA-4 and a tumor cell vaccine. Interestingly, similarly treated, non-transgenic mice developed a destructive prostatitis. Taken together, our findings demonstrate the potency of this approach: by combining these synergistic therapies, both a potent anti-tumor and autoimmune response can occur. Future studies will address the link between tumor immunity and autoimmunity.

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FOREWORD

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Introduction

Generation of an effective T cell response requires both an antigen-specific signal through the T cell antigen receptor complex and an antigen-independent costimulatory signal through CD28 ligation by B7 on an antigen presenting cell. Upon activation, T cells express a second B7 receptor, CTLA-4. Unlike CD28, however, CTLA-4 delivers an inhibitory signal. Antibody-mediated blockade of CTLA-4 *in vivo* results in enhanced T cell responses in murine models of autoimmunity and tumor challenge (4-6). The goal of this project is to apply our understanding of the mechanisms of T cell activation to the immunotherapy of mammary carcinoma. Our initial studies using a transplantable mammary carcinoma demonstrated that treatment of mice with anti-CTLA-4 and a cell-based vaccine modified to express granulocyte-macrophage colony-stimulating factor (GM-CSF, a cytokine that promotes the growth and differentiation of dendritic cells, a potent antigen presenting cell population), was effective at treating recently established subcutaneous tumors. To further those studies, we used this treatment regimen on an experimental model of mutagen-induced mammary carcinogenesis. Our initial findings suggested that vaccination of mice that rejected mammary tumor cells were partially protected from mutagenesis. This past year, we extended these findings in both a transplantable melanoma model and a transgenic model of primary prostate carcinogenesis.

Body

Our initial studies using the N-methyl-N-nitrosourea (MNU) mutagenesis system suggested that rejection of SM1 might protect against subsequent mutagenic treatment. However, those studies suffered from a somewhat lower-than-anticipated tumor incidence. In final count, only 72% mammary tumor incidence was observed in control mice; our collaborators had suggested that incidence would be closer to 100% (7). In addition, little effect of the combination of the GM-CSF-expressing vaccine and anti-CTLA-4 on tumor incidence was observed. As reported last year, only mice that had rejected a B7/interferon-γ-expressing SM1 tumor had reduced primary tumor incidence. We have subsequently learned from Dr. Nandi's laboratory that SM1 was generated by *in vitro* mutagenesis of a pre-neoplastic line with MNU; this raised the concern that the antigen profile might not be shared with tumors arising in mutagenized mice. Therefore, we generated new tumor lines from mutagenized mice to replace the SM1 tumors as vaccines and transduced them to express GM-CSF. Unfortunately, at this time, mouse parvovirus was detected in our mouse colony. Mouse parvovirus is known to have adverse effects on the immune system. In consideration with the low incidence of tumors in our first experiment and the high cost of performing a large scale experiment that might generate uninterpretable data, this viral infection forced us to postpone further experiments of this nature.

At that time, we had initiated studies with Dr. Norman Greenberg (Baylor College of Medicine) and Dr. Eugene Kwon (Loyola College of Medicine) using a transgenic model of prostate cancer developed in the Greenberg Lab. **TR**ansgenic **A**denocarcinoma of the **M**ouse **P**rostate (TRAMP) mice express an

SV40 T antigen transgene under the transcriptional control of the rat probasin promoter. Expression of the oncogene is restricted to prostatic epithelium in an androgen-regulated manner. Pathogenesis of neoplasia in TRAMP mice mirrors that in man. Over time, male TRAMP mice develop hyperplasia (5-8 weeks of age), frank neoplasia (8-12 weeks)and eventually invasive adenocarcinoma with metastasis to the lungs, lymph nodes and bone (15-20 weeks). As reported last year, we had undertaken an initial experiment of treating TRAMP mice with anti-CTLA-4 in combination with a prostate cancer cell-based vaccine (TRAMPC) modified to express GM-CSF. Groups of 30 mice were treated at approximately 15 weeks of age and tumor incidence was assessed at 8 weeks after treatment. In addition, histopathological analysis was performed on the prostatic complex to assess the tumor grade of prostatic lesions. We observed a significant decrease in tumor incidence in mice treated with anti-CTLA-4 and either a control vaccine (TRAMPC, 43%, $P<.05$) or a GM-CSF-expressing vaccine (GMTRAMPC, 33%, $P<.01$) when compared to untreated mice.

Because mice within each group were born at two different points, two weeks apart, tumor incidence was reassessed as a function of age. These analyses revealed that the significant reduction in tumor incidence was in the mice treated at 14 weeks of age ($p=.003$, figure 2) and not in the group treated at 16 weeks of age ($p=.1$). This finding suggests that there was a distinct time when therapy of TRAMP mice was effective. However, histologic analysis did not reveal any gross differences in the pathology of prostatic lesions between TRAMP mice treated at 14 and 16 weeks of age. This suggests that there was a distinct time when therapy of TRAMP mice was effective. However, histologic analysis did not reveal any gross differences in the pathology of prostatic lesions between TRAMP mice at 14 and 16 weeks of age.

Histopathological analyses also revealed the effectiveness of these treatments. Statistical analyses revealed a significant reduction in the severity of lesion in mice treated with anti-CTLA-4 and either vaccine. Specifically, TRAMP mice vaccinated with TRAMPC (mean peak score 4.6) had a significantly lower score than sham-treated (control Ig/no vaccine) mice (mean peak score 5.5, $p=.03$). Even more striking was the finding that mice vaccinated with GMTRAMPC has a significantly lower score (mean peak score 3.9) than all three control groups: sham ($p=.0009$), control Ig/GMTRAMPC (mean peak score 5.5, $p=.0002$), and anti-CTLA-4 treatment alone (mean peak score 4.8, $p=.04$). Similar to their effect on tumor incidence, treatment with either vaccine without CTLA-4 blockade had no significant effect on the histological severity of prostatic neoplasia. These findings imply that in addition to reducing the incidence of primary tumors, vaccination with a tumor cell-based vaccine in combination with CTLA-4 blockade can lessen the severity of prostatic lesions in TRAMP mice.

The most striking histologic feature of the treated TRAMP mice was observed in mice treated with GMTRAMPC and anti-CTLA-4. In these animals, there was a significant accumulation of inflammatory cells in the interductal spaces. In these mice, inflammatory cells were closely associated with the vasculature found in the stroma. In contrast, there was no detectable accumulation of inflammatory cells in any of the sham-treated mice. These findings indicated

that this treatment regimen was inducing a moderate inflammatory prostatitis. Interestingly, the TRAMPC vaccines do not express the SV40 TAg. To confirm that the elicited response was directed against normal prostate antigens, non-transgenic (wild-type) mice were similarly vaccinated. Histopathological analysis of 12 week-old male mice vaccinated with GMTRAMPC and treated with anti-CTLA-4 revealed significant destruction of prostatic epithelium in the dorsolateral prostate of some mice 28 days after sensitization, as compared to unvaccinated prostate. At later time points (6 and 8 weeks after sensitization), less infiltration and little destruction of glandular structures was noted. Studies planned for my new laboratory are addressing the mechanism by which the immune system may overcome tolerance to peripheral, tissue-specific antigens, as a way to develop a more potent anti-tumor response.

In a separate study conducted with Dr. Andrea van Elsas in Dr. Allison's laboratory, we used a similar approach to treat murine melanoma. The B16 melanoma is an aggressive, pigmented melanoma considered to be poorly immunogenic. We have demonstrated that similar to the poorly immunogenic SM1 mammary carcinoma (8), neither anti-CTLA-4 nor a GM-CSF-expressing vaccine was effective at treating B16. However, treatment of subcutaneous B16 tumors up to eight days after implantation with both regimens resulted in tumor regression and immunity to rechallenge. Moreover, this approach was effective at treating lung metastases as well. Interestingly, unlike the SM1 model, tumor rejection was only dependent on CD8+ cells and not CD4+ cells, suggesting that direct induction of CD8+ T cells in the absence of CD4-mediated help is possible.

The most remarkable feature of this model is that about half of mice that rejected the B16 melanoma underwent a progressive autoimmune depigmentation that started at the site of vaccination and progressed across the coat of the mice. Subsequent studies suggest that B cells may also contribute to the depigmentation: we have observed antibody deposition adjacent to the sites of pigment extrusion in the hair follicles that is not observed in unaffected areas of the coat. This depigmentation is reminiscent of the vitiligo syndrome observed in melanoma patients and confirms the potency of the vaccination regimen. In my new lab, we will study the mechanism of this depigmentation by looking at tolerance to various melanocyte-related antigens.

I think it is important to note that I have also been involved (actually, led) a separate research project looking at the role of T cell activation in autoimmune demyelination using experimental allergic encephalomyelitis (EAE), an animal model for the human disease, multiple sclerosis. We previously showed that blockade of CTLA-4 during sensitization to myelin antigen results in exacerbated disease pathogenesis in susceptible strains of mice (5). This past year, we have observed that in mice considered to be resistant to disease induction, CTLA-4 blockade results in disease induction. This appears to be the consequence of increased interferon-γ production by T cells. These studies will form a bridge between future studies on tumor immunity and autoimmunity. Similar studies will be pursued in my lab.

Taken together, these studies begin to address the relationship between the autoimmune and anti-tumor immune responses. In both the TRAMP model and the B16 model, a successful anti-tumor response was associated with a tissue-specific autoimmune response. As mentioned above, my new laboratory will focus on understanding how the immune system can be modulated to overcome its tolerance to self-antigens to generate a tissue-specific, anti-tumor immune response. In 'non-vital' tissues, this approach may be the key to inducing a long-lasting anti-tumor response without severe side effects. In addition, my laboratory will also study the combination of immunotherapy as an adjunctive therapy with anti-angiogenesis and androgen ablation therapy in the TRAMP model. If possible, we will attempt to address the ability of this immunotherapeutic strategy to treat primary mammary carcinoma using the MNU mutagenesis model described in the proposal. However, given the lower frequency of tumors and the length of time to tumor formation (6-12 months), further testing of this model will be necessary. Additional models for studying immunotherapy of mammary tumors are being sought and a include potential collaboration with Dr. Susan Ostrand-Rosenberg using the BALB/C-derived 4T1 mammary carcinoma (9).

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Appendix

Accomplishments:

-Demonstrated successful immunotherapy of primary prostate cancer in TRAMP mice

-Demonstrated rejection of melanoma results in depigmentation syndrome
-this may be mediated by both T and B cells

-In EAE, a murine model of multiple sclerosis, CTLA-4 blockade resulted in induction of clinical and histologic disease in 'resistant' strains of mice

Reportable Outcomes

van Elsas, A., A.A. Hurwitz, and J.P. Allison. 1999. Combination immunotherapy of B16 melanoma using anti-CTLA-4 and GM-CSF-producing vaccines induces rejection of subcutaneous and metastatic tumors accompanied by autoimmune depigmentation. *J Exp Med* 190:355-366.

Hurwitz, A.A., B.A. Foster, E.K. Kwon, T. Truong, E.M. Choi, N.M. Greenberg, M.B. Burg, and J.P. Allison. 1999. Successful Immunotherapy of Autochthonous Primary Prostate Cancer Using a Combination of CTLA-4 Blockade and a GM-CSF-expressing Vaccine. *manuscript submitted.*

Arthur A. Hurwitz, Barbara A. Foster, Eugene D. Kwon, Norman M. Greenberg, and James P. Allison. Immunotherapy for Prostate Cancer in the Autochthonous TRAMP Model Using a Combination of a GM-CSF-Expressing Tumor Vaccine and CTLA-4 Blockade. Oral presentation at AACR meeting entitled "New Researched Approaches in the Prevention and Treatment of Prostate Cancer" in Palm Springs, CA, November, 1998.

A.A. Hurwitz, B.A. Foster, E.D. Kwon, N.M. Greenberg, and J.P. Allison. Immunotherapy for Prostate Cancer in the TRAMP Model Using a GM-CSF-Expressing Vaccine and CTLA-4 Blockade. Oral presentation at American Association of Immunologists' (FASEB) annual meeting, Washington, DC, April, 1999.

"T Cell Activation in the Autoimmune and Anti-Tumor Immune Response". Plenary lecture presented at Pezcoller Symposium, Rovereto, Italy, June, 1999.

I applied for and was granted a CaP CURE Young Investigator Award for studying T cell activation in the anti-prostate cancer response (3 year award, $50,000/year). Earlier in the funding year, I was a co-investigator with Dr. Allison on a one year grant for $150,000 from CaP CURE to study immunotherapy of prostate cancer.

I was actively involved in a job search for a faculty position from October, 1998 until March, 1999. I accepted an offer from SUNY Health Science Center and recently started a tenure track position (Assistant Professor) in the Department of Microbiology and Immunology.

See attached for the reprints

Immunotherapy for Prostate Cancer in the Autochthonous TRAMP Model Using a Combination of a GM-CSF-Expressing Tumor Vaccine and CTLA-4 Blockade.

Arthur A. Hurwitz, Barbara A. Foster, Eugene D. Kwon, Norman M. Greenberg, and James P. Allison. University of CA, Berkeley, CA; Baylor College of Medicine, Houston, TX; Loyola Univ Med Ctr, Maywood, IL.

Recent advances in the understanding of T cell activation have provided a basis for designing immunotherapeutic approaches to treating cancer. Efficient T cell activation is dependent on both antigen-specific T cell receptor engagement as well as antigen-independent costimulatory signals mediated through CD28 interaction with B7 on the antigen presenting cells. CTLA-4 is a second ligand for B7 expressed on T cells that unlike CD28, delivers an inhibitory signal. To determine whether the manipulation of costimulatory signals could be used as immunotherapy for prostate cancer, we have taken advantage of the autochthonous TRansgenic Adenocarcinoma of the Mouse Prostate (TRAMP) model. In this model, expression of the SV40 large T antigen is under the transcriptional control of the rat probasin promoter and is therefore restricted to the epithelial cells of the prostate. Male TRAMP mice spontaneously develop metastatic prostate cancer that closely reflects the pathogenesis observed in man. We previously demonstrated that blockade of CTLA-4/B7 interactions is sufficient to promote rejection of two TRAMP prostate tumor derived cell lines (TRAMPC1 and TRAMPC2) implanted into syngeneic, non-transgenic C57Bl/6 mice. In this study, TRAMP mice were treated with an irradiated, whole cell vaccine consisting of TRAMPC1 and TRAMPC2 cells transduced to express granulocyte-macrophage colony-stimulating factor (GM-CSF) in combination with CTLA-4 blockade using an antibody prepared against murine CTLA-4. Treatment was initiated when mice were 14-16 weeks old; previous histopathologic studies demonstrated that at this age, most TRAMP mice would exhibit signs of prostatic intraepithelial neoplasia (PIN) or early signs of cribiform structures. Mice (25/group) were euthanized 8 weeks after treatment. Each mouse was scored for prostate weight and the presence of tumor (primary and metastasis) at gross necropsy. Prostatic tissues were microdissected and processed for histological grading. Most strikingly, our results indicate that there was a significant reduction in tumor incidence at necropsy in mice treated with GM-CSF-expressing cells and anti-CTLA-4 compared to mice treated with control antibody alone ($P=.009$) or control antibody and GM-CSF-expressing cells ($P=.002$). Moreover, there was a significant reduction in the histologic progression of prostatic disease in mice treated with the GM-CSF-expressing vaccine and anti-CTLA-4 ($P=.003$). There was no significant difference in prostate weight, presumably due to the rapid growth of the tumors. There was also no significant difference in the rate of metastasis between the various treatment groups. In a parallel study, we have observed increased survival of TRAMP mice receiving a cell-based vaccine and anti-CTLA-4. The findings of these studies underscore two important ideas: 1) synergy between tumor-derived GM-CSF expression and CTLA-4 blockade is the most potent anti-tumor vaccination regimen tested and 2) immunotherapy is a feasible approach to treating prostate cancer. Additional studies in progress are exploring other vaccination approaches to elicit a more potent anti-tumor response.

Immunotherapy for Prostate Cancer in the TRAMP Model Using a GM-CSF-Expressing Vaccine and CTLA-4 Blockade. A.A. Hurwitz, B.A. Foster, E.D. Kwon, N.M. Greenberg, and J.P. Allison. Univ CA, Berkeley

Recent advances in the understanding of T cell activation have provided a basis for designing immunotherapeutic approaches to cancer. We have shown that blockade of CTLA-4/B7 interactions can promote regression of transplantable tumors (TRAMPC) derived from TRansgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mice that express SV40 large T antigen regulated by a prostate-specific promoter. In this study, TRAMP mice were treated with anti-CTLA-4 in combination with an irradiated, whole-cell vaccine consisting of TRAMPC cells transduced to express granulocyte-macrophage colony-stimulating factor (GM-TRAMPC). Mice were euthanized 8 weeks later, tumor incidence assessed and prostatic tissues microdissected for histological analysis. Our results indicate a significant reduction in tumor incidence in mice treated with GM-TRAMPC and anti-CTLA-4 compared to mice treated with control antibody alone (P=.009) or control antibody and GM-TRAMPC (P=.002). Moreover, there was a significant reduction in the histologic progression of prostatic disease in mice treated with the GM-TRAMPC vaccine and anti-CTLA-4 (P=.003). In a parallel study, we observed increased survival of TRAMP mice receiving a cell-based vaccine and anti-CTLA-4. The findings of these studies underscore two ideas: 1) synergy between vaccine-derived GM-CSF expression and CTLA-4 blockade is a potent anti-tumor therapy and 2) immunotherapy is a feasible approach to treating prostate cancer. On-going studies are addressing the ability of prostate-specific antigens to elicit a more powerful anti-tumor response as well as the synergy between androgen ablation therapy and immunotherapy.

Combination Immunotherapy of B16 Melanoma Using Anti-Cytotoxic T Lymphocyte-associated Antigen 4 (CTLA-4) and Granulocyte/Macrophage Colony-Stimulating Factor (GM-CSF)-producing Vaccines Induces Rejection of Subcutaneous and Metastatic Tumors Accompanied by Autoimmune Depigmentation

By Andrea van Elsas, Arthur A. Hurwitz, and James P. Allison

From the Howard Hughes Medical Institute, Cancer Research Laboratory and Department of Molecular and Cellular Biology, University of California, Berkeley, California 94720-3200

Summary

We examined the effectiveness of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) blockade, alone or in combination with a granulocyte/macrophage colony-stimulating factor (GM-CSF)-expressing tumor cell vaccine, on rejection of the highly tumorigenic, poorly immunogenic murine melanoma B16-BL6. Recently established tumors could be eradicated in 80% (68/85) of the cases using combination treatment, whereas each treatment by itself showed little or no effect. Tumor rejection was dependent on $CD8^+$ and $NK1.1^+$ cells but occurred irrespective of the presence of $CD4^+$ T cells. Mice surviving a primary challenge rejected a secondary challenge with B16-BL6 or the parental B16-F0 line. The same treatment regimen was found to be therapeutically effective against outgrowth of preestablished B16-F10 lung metastases, inducing long-term survival. Of all mice surviving B16-BL6 or B16-F10 tumors after combination treatment, 56% (38/68) developed depigmentation, starting at the site of vaccination or challenge and in most cases progressing to distant locations. Depigmentation was found to occur in CD4-depleted mice, strongly suggesting that the effect was mediated by CTLs. This study shows that CTLA-4 blockade provides a powerful tool to enhance T cell activation and memory against a poorly immunogenic spontaneous murine tumor and that this may involve recruitment of autoreactive T cells.

Key words: CTLA-4 • immunotherapy • autoimmunity • vitiligo • melanoma

Recent work has shown that unaltered self-antigens aberrantly expressed in tumors or expressed in a tissue-specific fashion can be recognized by T cells isolated from mice or human cancer patients (for review see references 1 and 2). This finding suggests that autoreactive T cells escape thymic deletion and reach the periphery, where they can in some instances be activated and involved in antitumor immune responses. It is generally believed that these autoreactive T cells display relatively low avidity (3) but can be effective when activated under proper circumstances (4–7). In addition to the characterization of these self-antigens targeted in antitumor responses, our understanding of the requirements for proper T cell activation have provided possible explanations for the absence of tumor-specific immunity.

Full activation of naive T cells requires stimulation of the TCRs by corresponding peptide–MHC complexes, as well as costimulation through engagement of CD28 by B7.1 or B7.2 (B7) on the APCs (for review see reference 8). Stimulation of T cells by antigen in the absence of costimulatory signals can result in unproductive T cell stimulation or T cell tolerance (9). The lack of expression of B7 by tumor cells was shown to be one factor that can contribute to their failure to elicit productive immune responses (10, 11). CTLA-4 is a second counterreceptor for B7 that plays an inhibitory role in T cell activation. Accumulating data suggests that CTLA-4 engagement downregulates T cell responses by raising the threshold of signals needed for effective T cell activation, although it is possible that CTLA-4 might also play a role in terminating ongoing T cell responses (12). In vivo, monoclonal antibodies that block CTLA-4/B7 interactions have been shown to enhance $CD4^+$ T cell expansion in response to a variety of stimuli, including peptide antigens, superantigen, and parasites, and can exacerbate and accelerate autoimmune disease in murine models of diabetes and experimental autoimmune encephalitis (for review see reference 12). It has been reported that blockade of CTLA-4/B7 interactions prevents induction of peripheral T cell tolerance upon vaccination with peptides under

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tolerogenic conditions, suggesting that CTLA-4 might be actively involved in the induction of anergy (13).

We have previously shown that CTLA-4–blocking antibodies accelerate rejection of B7-transfected tumor cells and can induce rejection of large, established B7-negative tumors (14). When applied to a variety of tumor models, we found that susceptibility to anti–CTLA-4–induced rejection correlated with susceptibility to B7-induced rejection (Leach, D.R., manuscript in preparation; reference 15). This suggests that susceptibility to CTLA-4–induced regression is related to the inherent immunogenicity of the tumor. Thus, immunogenic tumors such as the fibrosarcoma Sa1/N, 51BLim10, RENCA, and the prostate carcinoma TRAMP/C1 were completely rejected by injection of CTLA-4–blocking antibodies, whereas outgrowth of poorly immunogenic tumors such as the melanoma B16-BL6 or the mammary tumor SM1 was minimally affected (14, 16; Leach, D.R., manuscript in preparation). Synergy with a GM-CSF tumor cell vaccine was demonstrated in the case of the SM1 tumor (17). Although these studies did not directly demonstrate enhanced tumor-specific T cell activity as a result of CTLA-4 blockade, in vivo depletion experiments demonstrated that both $CD4^+$ and $CD8^+$ T cells were required for rejection of the immunogenic tumors 51BLim10, Sa1/N, and SM1 (17). $NK1.1^+$ cells were found to also play an intriguing but not yet defined role in the eradication of TRAMP/C1 by CTLA-4 (Hurwitz, A.A. and J.P. Allison, unpublished observations).

In this study, we show that the combination of CTLA-4 blockade and GM-CSF–producing vaccines is therapeutically effective against the highly tumorigenic and poorly immunogenic melanoma B16-BL6 in a mechanism dependent on $CD8^+$ and $NK1.1^+$ cells but independent of $CD4^+$ T cells. Mice cured from established subcutaneous B16-BL6 tumors are immune to rechallenge with B16-BL6 or the parental line B16-F0 after 4 mo. We further show that B16-F10 pulmonary metastases can be eradicated by the combination treatment and that metastatic lesions from these mice show extensive infiltration by mononuclear cells. In both the subcutaneous and metastatic melanoma models, we found that surviving mice developed depigmentation, indicating that autoimmunity directed against pigmented cells was concurrently induced. As animals depleted of $CD4^+$ T cells also developed depigmentation, it is very likely that this autoimmune phenomenon is induced by $CD8^+$ T cells directed against pigmentation antigens. This model is well suited to studying the significance of autoreactive $CD8^+$ T cells in antitumor responses as well as investigating the role of CTLA-4 in peripheral tolerance in a preclinical setting relevant to the immunotherapy of cancer.

Materials and Methods

Mice. C57BL/6 female mice (obtained from Charles River Labs/National Cancer Institute) were maintained and treated in accordance with National Institutes of Health and American Association of Laboratory Animal Care regulations and used for tumor experiments when 8–12 wk old. All subcutaneous injections were performed after mice inhaled of the anaesthetic methoxyflurane.

Antibodies. Generation and purification of the hamster anti-murine CTLA-4 antibody 9H10 has been described in previous work (18). Similarly, GK1.5 (anti-CD4), 2.43 (CD8), PK136 (NK1.1), and 116.3 (Lyt2.1; rat IgG, obtained from B.J. Fowlkes, National Institute of Allergy and Infectious Diseases, Bethesda, MD) were prepared in our laboratory as ascites or purified from supernatant using standard procedures. Mouse IgG and hamster IgG were purchased from Jackson ImmunoResearch Labs., Inc., and rat IgG was from Sigma Chemical Co. RM4.4–PE (CD4), anti-CD8b2–PE, and DX5 (pan-NK) were obtained from PharMingen and were used to confirm depletions of the relevant population.

Cell Lines and GM-CSF Gene Transduction. B16-BL6, B16-F10 (obtained from Dr. I. Fidler, MD Anderson Cancer Center, Houston, TX), B16-F0 (American Type Culture Collection), and DC2.4 (19) were cultured in DMEM supplemented with 1 U/ml penicillin, 1 μg/ml streptomycin, 50 μg/ml gentamycin, 2 μM L-glutamine, and 8% FCS (hereafter referred to as complete DMEM). The C57Bl/6-derived tumor cell lines EL4 (thymoma) and MC38 (colorectal carcinoma; obtained from Dr. N. Restifo, National Cancer Institute, Bethesda, MD) were maintained in RPMI supplemented with antibiotics, L-glutamine, 20 μM β-ME, and 8% FCS. GM-CSF–producing B16-BL6 and B16-F10 were obtained by retroviral transduction (20). GM-CSF production by short-term lines (F10) or clones (BL6) was tested by ELISA using commercially available antibodies to murine GM-CSF (PharMingen). Clones BL6/GM-E, BL6/GM-18, BL6/GM-45, BL6/GM-52 (producing 5, 20, 40, or 50 ng GM-CSF/10^6 cells/24 h, respectively), and the line F10/g (producing 30–40 ng/10^6 cells/24 h) were cultured using complete DMEM. GM-CSF production was routinely confirmed in vitro during the course of vaccination experiments.

Subcutaneous Challenge and Treatment Experiments. Mice were shaved on the back and challenged subcutaneously with 10^4 B16-BL6 cells in PBS. At the same day or later as indicated, treatment was initiated by injecting 10^6 irradiated (16,000 rads) GM-CSF–producing cells (in PBS) subcutaneously into the left flank and repeated 3 and 6 d later. The vaccine consisted of a 1:1 mixture of clones BL6/GM-E and BL6/GM-18. Treatment with 9H10 or control hamster IgG was started simultaneously or 3 d later with similar results. Antibodies were delivered intraperitoneally at 100 μg in PBS, usually followed by two 50-μg injections every 3 d. Tumor growth was scored by measuring perpendicular diameters. Mice were killed when the tumors displayed severe ulceration or reached a size of 300 mm^2. Depletion of T or NK cells was accomplished by injection of the relevant antibodies (500 μg, i.p.) 7, 6, and 5 d before tumor challenge and maintained by injections every 10 d during the experiment. Depletions were confirmed in lymph nodes and spleens 1 d before challenge by flow cytometry using noncross-reactive antibodies. Routinely, <1% $CD4^+$ T cells, $CD8^+$ T cells, or $NK1.1^+$ cells were detected in lymph nodes (after CD4 or CD8 depletion) or spleens (NK1.1 depletion), whereas mice treated with control antibodies (mouse IgG, rat IgG, or 116.3) demonstrated unchanged lymphocyte profiles as compared with untreated mice.

Treatment of Lung Metastases. To establish lung metastases, mice were injected intravenously with 5×10^4 or 10^5 B16-F10 cells. Treatment using irradiated F10/g cells and antibodies was started after 24 h, following the same protocol as outlined for treatment of subcutaneous tumors. After 25 d, lungs were harvested from each treatment group and surface metastases were counted using

a dissection microscope. Paraffin-embedded lung sections were stained with hematoxylin–eosin using standard procedures. For survival experiments, 5×10^4 B16-F10 cells were injected intravenously and treatment was started the next day.

Generation of CTL Cultures and IFN-γ Release Assay. Spleens were harvested from mice rejecting B16-BL6 and restimulated in vitro with B16-BL6/B7.1 or a mixture of B16-F0 and the dendritic cell line DC2.4 after overnight coculture. 5×10^6 spleen cells were mixed with 10^5 irradiated (16,000 rads) stimulator cells, and recombinant human IL-2 was added to a final concentration of 30 IU/ml. After 7 d, cells were collected and purified by Histopaque (Sigma-Aldrich) gradient centrifugation. Live cells (2.5×10^5 per well) were stimulated with target cells (5×10^4 per well) in 96-well round-bottom plates for 24 h, after which supernatant was collected and tested for the presence of IFN-γ by sandwich ELISA (PharMingen).

Results

CTLA-4 Blockade Together with GM-CSF–producing Cellular Vaccines Causes Rejection of Established B16-BL6 Tumors. B16-BL6 was originally derived from the spontaneous murine melanoma cell line B16-F0 by in vivo selection for invasiveness (21). Both the parental line and its variant express low levels of H-2K^b and D^b, and MHC class II is undetectable by flow cytometry in vitro and ex vivo (data not shown). Vaccination with irradiated B16-BL6 does not protect against subsequent challenge with live B16-BL6 cells, nor does B7.1 expression result in any significant change in tumor growth in vivo (20, 22; our unpublished results). By these criteria, B16-BL6 is a very poorly immunogenic tumor. In previous experiments, we had found that CTLA-4 blockade was not therapeutically effective against poorly immunogenic tumors such as B16-BL6. We also found that vaccination with irradiated B16-BL6 cells in combination with anti–CTLA-4 was ineffective (data not shown). We hypothesized that this might be due to insufficient presentation of tumor antigens by host APCs. Therefore, we chose to combine CTLA-4 blockade with GM-CSF–producing irradiated B16-BL6 whole cell vaccine, which was described by others as the most effective prophylactic vaccine against B16 (20) and augmented immunity against SM1 (17). Presumably, GM-CSF production at the site of vaccination might attract host APCs and enhance their function in vivo. C57BL/6 mice were challenged with 10^4 B16-BL6 cells subcutaneously and subsequently treated starting on the same day or 4–12 d later. A representative experiment is shown in Fig. 1. Administration of anti–CTLA-4 antibody 9H10 or control hamster IgG by themselves had no effect on growth of B16-BL6 tumors. Vaccination with irradiated GM-CSF–producing B16-BL6 cells along with control antibody delayed growth when initiated at the time of tumor implantation but had no effect when treatment was delayed. However, the combination of GM-CSF–producing vaccine and CTLA-4 blockade induced rejection of all tumors injected the same day or 4 d earlier. One of five mice carrying a day 8 B16-BL6 tumor rejected a small palpable tumor after combination treatment including CTLA-4 blockade. The growth of tumors established 12 d earlier was also delayed by the combination treatment, although rejection was not obtained. When the data from a series of 10 experiments were combined, an overall success rate of combination treatment of 80% was achieved (68/85 mice cured) when treatment was begun at day 0 or 4 d after tumor implantation (Table I). These results corroborate the finding that CTLA-4 blockade and GM-CSF–producing vaccines act synergistically to cause rejection of poorly immunogenic tumors (17).

A single dose of GM-CSF–producing vaccine administered on the same day as tumor challenge was sufficient to eradicate tumors in all of the mice when combined with CTLA-4 blockade (Fig. 2). Similarly, a single dose of anti–CTLA-4 after three vaccinations with GM-CSF–producing cells was sufficient to induce B16-BL6 rejection (not shown). GM-CSF production by the vaccine was found to

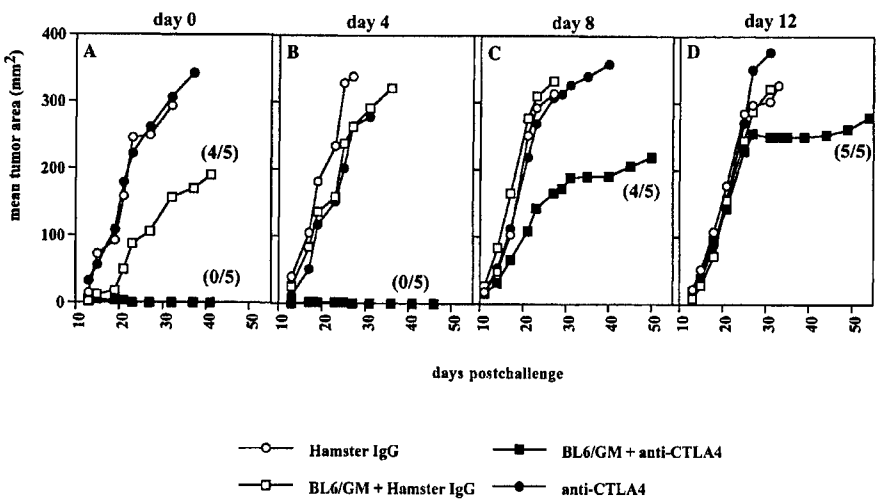

Figure 1. Successful treatment of preestablished B16-BL6 using anti–CTLA-4 and GM-CSF–producing BL6 vaccine. C57BL/6 female mice (five per group) were injected with 10^4 B16-BL6 cells subcutaneously on the back, on the same day (A) or 4, 8, or 12 d (B–D) before treatment was started. Treatment consisted of three consecutive injections (in a 6-d time frame as indicated in Materials and Methods) of anti–CTLA-4 antibody 9H10 intraperitoneally (●), control hamster IgG (100, 50, 50 μg; ○), or 10^6 irradiated BL6/g cells subcutaneously, in combination with 9H10 (■) or hamster IgG (□). Tumor growth (mm^2) was scored by measuring perpendicular diameters and was averaged for all mice within each group. In some treatment groups, only a fraction of the mice (indicated between brackets) developed a tumor.

Table I. *Combination Treatment of B16-BL6 Using Anti-CTLA-4 Plus GM-CSF-producing Vaccine*

Experiment no.	B16-BL6 Day of challenge	Treatment schedule*	Fraction of mice responding per treatment group				Depigmentation‡ (fraction of responding mice)
			Control IgG	Anti-CTLA-4	BL6/GM-CSF plus control IgG	BL6/GM-CSF plus anti-CTLA-4	
1	0	A	0/10	—	—	7/9	4/7
2	0	A	0/5	0/5	2/5	5/5	3/5
		B				5/5	3/5
		C				5/5	1/5
3	−4	A	0/5	0/5	0/5	3/5	1/3
4	0	A	0/5	—	0/5	6/8	3/6
		D			2/5	3/5	2/3
5	0	E	0/5	0/5	0/5	2/5	2/2
		F			2/5	3/5	2/3
6	0	A	0/5	—	—	6/10	4/6
7	0	A	0/5	0/5	1/5	4/5	1/4
8	0	A	0/5	0/5	0/5	9/9	6/9
9	−4	A	0/5	0/5	0/5	5/5	3/5
10	0	A	0/5	0/5	1/5	5/5	3/5
Total number of mice responding:			0/55	0/35	8/50§	68/85§	36/68
Percentage:			0	0	16	80	56

*Treatment regimen A: 10^6 irradiated GM-E plus GM-18, administered on days 0, 3, and 6 subcutaneously, plus 100 μg 9H10 or control hamster IgG (day 0) and 50 μg 9H10 (days 3 and 6); B: vaccine on days 0 and 3, then as in A; C: vaccine on day 0 only, then as in A; D: vaccine on days 0, 6, 20, 34, 48, 62, and 76, then rest as in A; E: clone GM-45 instead of GM-E plus GM-18; F: clone GM-52 instead of GM-E plus GM-18.
‡Depigmentation visible as outgrowth of nonpigmented coat, appearing between 4 and 8 wk after treatment.
§Responding fraction of mice is significantly different: $P < 0.000001$ (two-sided t test).

be critical for the synergistic effect, as vaccination with irradiated untransduced B16-BL6 cells in combination with anti-CTLA-4 antibodies was not effective, as had been found previously for synergistic treatment of SM1 (data not shown; reference 17).

Combination of CTLA-4 Blockade and GM-CSF-producing Vaccines Induces Effective Immunity to Rechallenge with B16-BL6. To determine whether mice cured from the initial challenge of B16-BL6 had developed immunity to rechallenge, surviving mice received a second challenge of 2×10^4

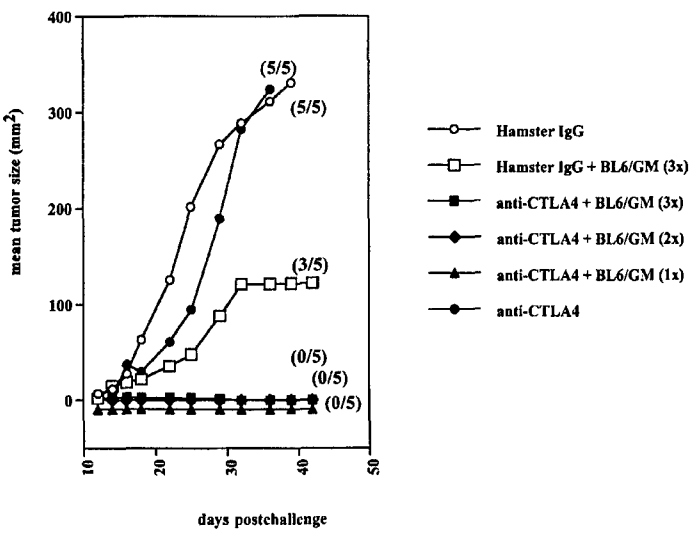

Figure 2. A single dose of GM-CSF-producing vaccine cooperates with CTLA-4 blockade to induce 100% cure of B16-BL6. Mice were inoculated subcutaneously with 10^4 B16-BL6 cells. On the same day, combination treatment was initiated using triple BL6/g vaccine (days 0, 3, and 6) combined with either hamster IgG (100, 50, 50 μg on days 3, 6, and 9; □) or anti-CTLA-4 (■). Control treatments consisted of antibody injections alone: hamster IgG (○) or anti-CTLA-4 (●). Also, anti-CTLA-4 treatment was combined with a single (▲) or double injection (◆) of the BL6/g vaccine. Average tumor size was calculated for all mice within a treatment group (mm^2). The fraction of mice developing tumors is shown between brackets.

B16-BL6 on the left flank 128 d after the primary challenge. Also, resistance to the parental B16-F0 melanoma cell line was tested by injecting 2×10^4 cells into the right flank. Naive age-matched control mice grew both tumors and required euthanasia within 30 d. All mice cured from a primary challenge with B16-BL6 rejected B16-F0. Within the first experiment, the two mice that had rejected the primary challenge after BL6/g vaccination alone were unable to reject a secondary B16-BL6 challenge (Table II). In contrast, seven out of nine mice that received BL6/g vaccine plus anti-CTLA-4 also rejected B16-BL6 (Table II). In two rechallenge experiments combined, 20/24 mice cured from B16-BL6 by combination treatment were immune to secondary challenge with B16-BL6, and 11 mice were resistant to rechallenge with B16-F0. Only four of eight mice cured upon vaccination with GM-CSF–producing cells alone were resistant to rechallenge, but the few mice surviving a primary tumor after treatment with BL6/GM-CSF vaccine alone did not allow any conclusion to be drawn as to the possible enhancement of memory formation by anti-CTLA-4 ($P = 0.062$, NS; Table II). Although immunity to rechallenge with B16-BL6 was not found in 100% of mice cured by the combination treatment, the fact that B16-F0 was rejected by all suggests that mice surviving a primary challenge with B16-BL6 had mounted adequate memory to an antigen(s) shared between the parental line and its more invasive variant.

$CD8^+$ and $NK1.1^+$ but not $CD4^+$ Cells Are Required for Combination Treatment of B16-BL6. To determine the involvement of T and NK cells in the rejection of B16-BL6, mice were depleted of $CD4^+$, $CD8^+$, or $NK1.1^+$ cells before tumor challenge. Treatment was started on the same day as tumor implantation following the general schedule of three simultaneous injections of vaccine and anti-CTLA-4. Depletion of $CD8^+$ cells abrogated the effect of treatment (Table III; $P = 0.017$ compared with control rat IgG). Mice depleted of $NK1.1^+$ cells were also largely unable to reject their tumors (8/10). We observed that the tumor-bearing, NK-depleted mice had developed multiple tumors at the site of challenge, suggesting that NK cells could be involved in the first line of defense against the MHC class I^{lo} B16-BL6 challenge. Surprisingly, $CD4^+$ T cells were not required for tumor rejection. In fact, 80% of the CD4-depleted mice rejected their tumors after treatment with anti-CTLA-4 and GM-CSF vaccine under suboptimal conditions where 50–60% of the control groups rejected B16-BL6 (Table III). Depletion of both $CD4^+$ and $CD8^+$ cells abolished the therapeutic effect. It is apparent that $CD8^+$ T cells and, most likely, $NK1.1^+$ cells are necessary for rejection of B16-BL6 using CTLA-4 blockade and GM-CSF–producing vaccines. Activation of $CD8^+$ T cells involved in rejection of B16-BL6 does not appear to be dependent on CD4 help in this system.

Table II. *Treatment of B16-BL6 Using Anti-CTLA-4 Facilitates Development of Memory to Rechallenge*

	Tumor incidence at secondary challenge	
Primary treatment	B16-BL6	B16-F0
Experiment 1 (rechallenge on day 128)		
BL6/GM plus hamster IgG	2/2	0/2
BL6/GM plus anti-CTLA-4	2/9*	0/9
None	5/5	5/5
Experiment 2 (rechallenge on day 100–130)		
BL6/GM plus hamster IgG	2/6	—
BL6/GM plus anti-CTLA-4	2/15*	—
None	5/5	—

*Two experiments combined: 20/24 mice protected from rechallenge after BL6/GM-CSF plus anti-CTLA-4, versus 4/8 after BL6/GM-CSF plus control IgG; P = 0.062 (NS; two-sided *t* test).

Table III. *Involvement of Lymphocyte Subsets in Rejection of B16-BL6 through Cotreatment with Anti-CTLA-4 and BL6/GM Vaccine*

Depletion	B16-BL6 tumor take*	Remarks
CD4	2/10‡§	Depigmentation (4/8 survivors)
CD8	9/10‖	—
CD4 plus CD8	5/5	—
NK1.1	8/10¶	Multiple tumors developed at injection site, no depigmentation
Control mouse IgG	5/10	Depigmentation (3/5 survivors)
Control rat IgG	4/10	Depigmentation (4/6 survivors)
No depletion	5/10	Depigmentation (3/5 survivors)
No depletion, no treatment†	10/10	—

Depletion of lymphocyte subsets was achieved by injecting depleting antibodies GK1.5 (anti-CD4), 2.43 (CD8), PK136 (NK1.1), or control antibodies at days −8, −7, −6, and every 7 (GK1.5) to 10 d thereafter. Depletion was checked at day −1. Results are compiled from two experiments.

*Live B16-BL6 challenge on day 0 was followed by anti-CTLA-4 and BL6/GM vaccination on days 0, 3, and 6.

‡Fraction of mice unable to reject the B16-BL6 challenge.

§CD4 depletion versus rat IgG: P = NS; CD4 versus CD8; $P = 0.00054$ (two-sided *t* test).

‖CD8 versus rat IgG: $P = 0.017$ (two-sided *t* test).

¶NK1.1 versus control mouse IgG: P = NS.

†Nondepleted mice were left untreated after challenge.

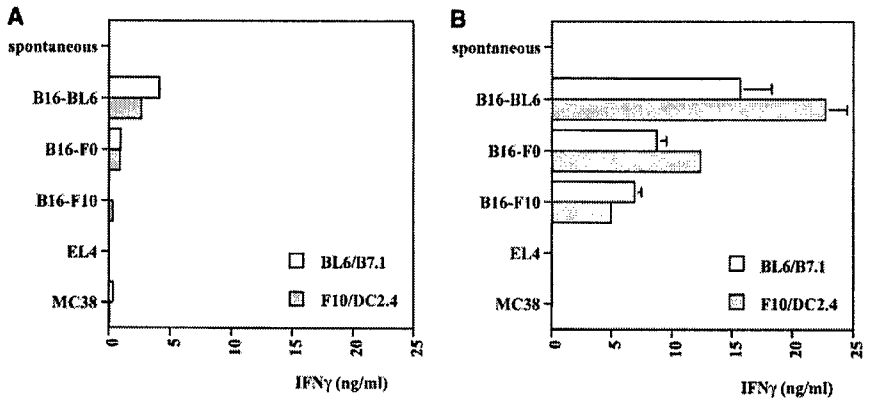

Figure 3. Anti-CTLA-4 enhances IFN-γ production by B16-specific T cells induced in vivo. Mice (four per group) were vaccinated with irradiated BL6/g (10^6 per mouse) and cotreated with control hamster IgG (A) or anti-CTLA-4 (B). After 4 wk, mice were challenged with 2×10^4 B16-BL6, and 10 d later, splenocytes were pooled and restimulated in vitro using B16-BL6/B7.1 (open bars) or a mixture of B16-F10 and DC2.4 dendritic cells (filled bars). On day 8, cultures were tested for tumor-specific IFN-γ release as described in Materials and Methods. Targets included B16 sublines -F0, -F10, and -BL6, as well as unrelated H-2^b tumors EL4 and MC38.

Generation of B16-specific T Cells Is Strongly Enhanced by CTLA-4 Blockade In Vivo. To determine if tumor-reactive T cells were induced by the combination therapy, mice were immunized with BL6/g plus anti-CTLA-4 or control IgG and challenged with B16-BL6 after 4 wk. 10 d after challenge, spleens from four mice in each group were pooled and restimulated with B16-BL6/B7.1 or a mixture of B16-F10 and the dendritic cell line DC2.4 (19). After one round of restimulation in vitro, specific IFN-γ release was tested using different variants of B16 and two unrelated tumor cell lines expressing the H-2^b haplotype, the thymoma EL4 and the colorectal carcinoma MC38. As shown in Fig. 3, T cells from mice vaccinated with BL6/g in the presence of control hamster IgG produced very low levels of IFN-γ in this assay. T cells from mice treated with anti-CTLA-4 in vivo had greatly enhanced B16-specific IFN-γ secretion. These results indicate that CTLA-4 blockade during vaccination with BL6/g specifically enhances reactivity toward an antigen (or antigens) expressed by B16 and its variants. In addition, all splenocyte cultures established from mice that were long-term (3–10 mo) survivors after combination treatment were found to specifically react with B16 and its variants, as tested by IFN-γ release after one round of restimulation in vitro (data not shown). Successful rejection of B16-BL6 coincides with the generation of tumor-specific T cell activity.

Table IV. *Reduced Number of B16-F10 Lung Metastases after Combination Treatment with Anti-CTLA-4 and F10/GM Vaccine*

Treatment of lung metastases	Lung metastasis count
Control hamster IgG	>200, >200, >200, 25, 16
Anti-CTL-4	>200, >200, >200, >200, >200
Hamster IgG plus F10/GM vaccine	>200, >200, 35, 49, 4
Anti-CTLA-4 plus F10/GM vaccine	87, 28, 6, 0, 0*

B16-F10 (10^5, i.v.)-induced lung metastases were treated with hamster IgG, 9H10, and F10/GM vaccine in combination with either antibody on days 1, 4, and 7 after challenge. Surface lung metastases were counted under a dissecting microscope 25 d after inoculation. Counts are shown for each individual mouse; all counts over 200 were scored as >200.
*F10/GM plus CTLA-4 versus control hamster IgG: $P = 0.057$ (NS; two-sided t test).

Suppression of B16-F10 Lung Metastases and Induction of Long-Term Survival by Combination Treatment. We next sought to determine whether anti-CTLA-4 combined with vaccination would be effective against metastatic disease. 10^5 B16-F10 cells (selected for metastasis exclusively to the lungs) were injected intravenously, and treatment was started 1 d later. On day 25, mice were killed and surface lung metastases were counted. Treatment with anti-CTLA-4 alone did not have any appreciable effect on the lung metastasis count as compared with control IgG (Table IV). Immunization with F10/g reduced the number of metastases in a few mice. Treatment of F10/g-vaccinated mice with anti-CTLA-4 further suppressed lung colonization and completely inhibited pulmonary metastases in two of five mice sampled. Histological analysis of these lung samples demonstrated that CTLA-4 blockade in combination with F10/g vaccination was associated with infiltration of mononuclear cells in all of the metastases stained and observed in three of the five tumor-bearing lungs (the two remaining sets of lungs were found to be tumor free) (Fig. 5). Neither anti-CTLA-4 nor F10/g vaccination alone resulted in lymphocytic infiltration in lung tumors or surrounding tissue. A few polymorphonuclear cells were observed in the smaller metastases from mice vaccinated with F10/g in the presence of control IgG, but there were no extensive infiltrates in larger lesions in any of the control groups. The observation that the combination therapy had at least some effect in enhancing infiltration and reducing lung metastases led us to test its effectiveness in increasing survival, as shown in Fig. 4. Mice challenged with 5×10^4 B16-F10 cells and treated with control hamster IgG all (10/10) succumbed to lung failure due to extensive metastatic disease by day 75 after injection. Anti-CTLA-4 by itself prolonged survival, as did vaccination with F10/GM. However, 13/13 mice receiving

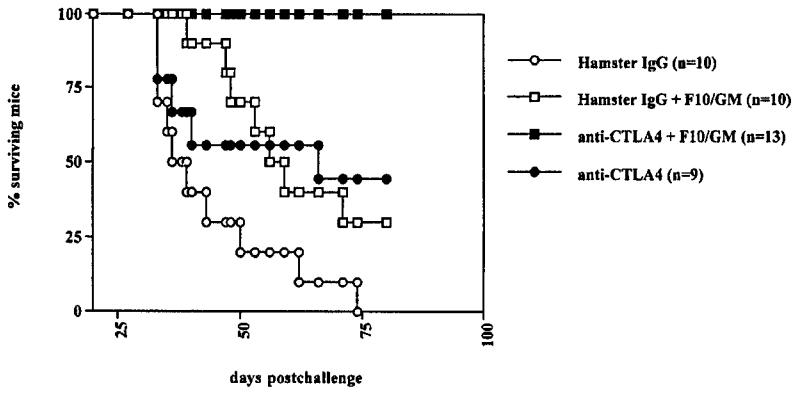

Figure 4. Mice bearing B16-F10 lung metastases show enhanced survival when treated with anti-CTLA-4 and F10/g vaccine. B16-F10 cells (5×10^4 per mouse) were injected into the tail vein and 24 h later, treatment was started using control hamster IgG (10 mice, ○), anti-CTLA-4 antibody 9H10 (9 mice; ●), irradiated F10/g (10^6 subcutaneously) in combination with hamster IgG (10 mice; □) or 9H10 (13 mice; ■) on days 1, 4, and 7, according to the dosing schedule used for subcutaneous tumors (see Fig. 1 legend). Mice were followed for survival, and in some subjects death due to extensive pulmonary metastasis was confirmed by harvesting lungs postmortem.

the combination treatment were still alive by day 80 (Fig. 4). Lungs taken from these surviving mice did not demonstrate metastatic lesions on their surfaces. This is the first demonstration that CTLA-4 blockade in vivo is therapeutically effective against disseminated disease.

Mice Surviving Subcutaneous B16-BL6 Tumors or B16-F10 Lung Metastases Develop Skin and Hair Depigmentation. Within 4–8 wk after challenge, 56% (38/68 cured mice) of the surviving mice developed depigmentation, starting at the sites of vaccination (left flank) and challenge (back) (Fig. 6 A). Moreover, depigmentation was observed at the site of vaccination in a similar proportion of mice surviving B16-F10 lung metastases (Fig. 6 B). Rejection of a B16-BL6 tumor established 8 d before start of treatment (Fig. 1) induced fast and progressive depigmentation appearing within 25 d after challenge and spreading to distant sites, indicating that a relatively strong antitumor response resulted in rapid manifestation of progressive depigmentation (Fig. 6 C). Depigmentation did occur in mice that received combination treatment in a prophylactic setting but at reduced frequency (not shown). Interestingly, depigmentation was not dependent on the presence of $CD4^+$ T cells, as four of eight CD4-depleted mice rejecting their tumors also developed progressive depigmentation (Table III). In some cases, tumor-bearing mice (moribund despite treatment with anti-CTLA-4 and BL6/GM) were found to develop small areas of hair depigmentation at the site of progressive tumor growth. Depigmentation was never observed in the mice that were treated by BL6/GM-CSF vaccination without CTLA-4 blockade or in any of the other treatment groups. These findings suggest that CTLA-4 blockade allows for the activation of autoreactive lymphoid cells that are involved in rejection of a tumor de-

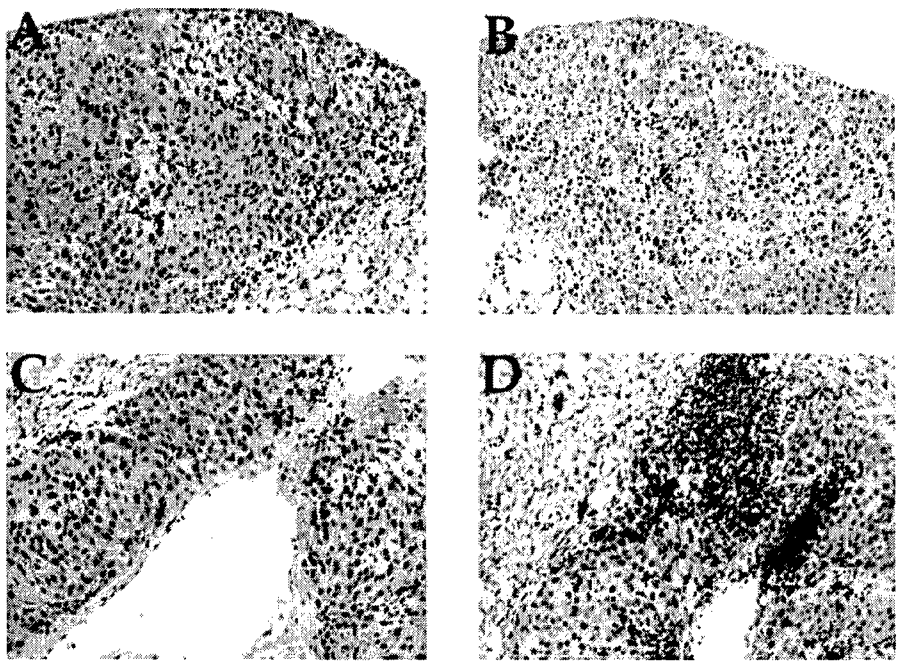

Figure 5. B16-F10 metastases demonstrate lymphocytic infiltration after treatment with anti-CTLA-4 and F10/g vaccine. Mice injected with 10^5 B16-F10 intravenously and treated with control hamster IgG (A), 9H10 (B), or F10/g vaccine in combination with either hamster IgG (C) or 9H10 (D) on days 1, 4, and 7, as outlined in the Fig. 4 legend. On day 25, lungs were harvested, fixed in 10% neutral-buffered formalin, and processed for hematoxylin–eosin staining.

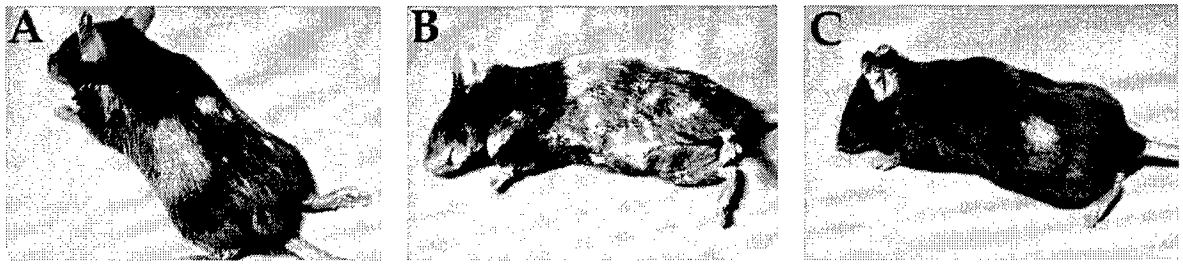

Figure 6. Rejection of B16-BL6 or B16-F10 as a result of treatment with anti-CTLA-4 and GM-CSF-producing vaccines causes autoimmune skin and hair depigmentation. After successful treatment for B16-BL6 subcutaneously or B16-F10 intravenously, C57Bl/6 mice developed skin and hair depigmentation. (A) Depigmentation of both sites of vaccination and challenge, after rejection of a day 0 tumor. (B) Progressive depigmentation found in a mouse rejecting a B16-BL6 subcutaneous tumor, established 8 d before treatment started. (C) Depigmentation at the site of vaccination of a mouse cured from preestablished B16-F10 lung metastases.

rived from the melanocytic lineage and may also mediate rejection of normal pigment-containing cells in the skin and hair follicles expressing pigmentation antigens.

Discussion

In this study, we show that administration of anti-CTLA-4 antibody, when combined with an irradiated GM-CSF-producing tumor cell vaccine, results in rejection of previously established primary tumors and resistance to secondary challenge in mice inoculated with the nonimmunogenic melanoma B16-BL6. Similarly, this combination treatment led to the eradication of B16-F10 lung metastases. The combination treatment induced massive infiltration of mononuclear cells into the remaining lung metastases. Tumor rejection by the combination treatment was reflected by an enhancement of B16-specific T cell responses in vitro. After tumor eradication, 56% of the surviving mice developed depigmentation of the hair (Table I). Both tumor rejection and subsequent depigmentation were dependent on the presence of $CD8^+$ T cells and $NK1.1^+$ cells but did not require $CD4^+$ T cells.

We have found that treatment with anti-CTLA-4 is sufficient to obtain rejection of many, but not all, experimental tumors (14, 16, 17). The effectiveness of CTLA-4 blockade appears to correlate with that of B7-positive tumor cell vaccines, suggesting that it is most effective against tumors with a significant degree of intrinsic immunogenicity. The lack of therapeutic effectiveness of CTLA-4 blockade by itself on B16-BL6 can most likely be attributed to the poor capacity of this tumor to provide antigens to host APCs. GM-CSF-transduced tumor cells have been shown to induce potent immunity to a variety of tumors, including B16 (20, 22). The effectiveness of GM-CSF in these systems can probably be attributed to the capacity of this cytokine to attract host bone marrow-derived APCs and enhance their differentiation, thereby increasing their capacity to capture tumor-derived antigens in the local environment of the irradiated tumor cell vaccine (23–25). Although this immunization strategy has been shown to greatly enhance the immunogenicity of B16 cells and leads to resistance to subsequent challenge with viable tumor cells, the response elicited by irradiated GM-CSF tumor cells is only marginally if at all effective in the treatment of preestablished tumors. We obtained tumor rejection in 16% of mice treated with the vaccine alone (Table I), and then only when it was administered on the same day as tumor challenge. These results suggest that GM-CSF vaccine has a limited potential to elicit an effector cell response of sufficient potency to obtain rejection in tumor-bearing mice. The potency of the combination of the vaccine and anti-CTLA-4 antibody can likely be attributed to enhanced cross-priming of T cells by host APCs by the vaccine, together with a highly potentiated T cell response as a result of the removal of the inhibitory effects of CTLA-4 by antibody blockade. This results in a synergistic enhancement of the T cell response to a level capable of eliminating the preexisting tumor cell mass. This could occur as a consequence of activation of a larger number of naive T cells due to a lowering of the threshold for activation or a more sustained response due to temporary removal of signals involved in terminating the response (12). Rejection is accompanied by long-lived memory, as indicated by the fact that cured mice reject rechallenge in the absence of treatment 4 mo after the initial treatment.

Whereas the combination treatment resulted in an overall cure rate of 80% in mice treated on day 4 or before, effectiveness was much lower when initiated at day 8 and was essentially ineffective at day 12 or later. This is in contrast to our previous findings that CTLA-4 blockade by itself was quite effective in the treatment of well established tumors in other model systems. Subcutaneous tumors of the colon carcinoma 51Blim10 or the fibrosarcoma SalN could be eradicated when antibody was administered beginning as late as 2 wk after tumor inoculation, and complete eradication was obtained even when treatment was delayed until the tumors reached a size of 100–140 mm^2 (Leach, D.R., manuscript in preparation). The difference in the responses obtained in these experiments and in this study may be related to the relative antigenicity of the systems—more immunogenic targets may also be better targets f or effector T cells than the poorly immunogenic B16-BL6, which might simply be able to outstrip the emerging T cell response. It may be that tumors grow beyond a critical size than can be effectively dealt with by the immune response. It is also possible that loss of effectiveness

of the vaccine is a consequence of induction of nonresponsiveness or tolerance in tumor-reactive T cells. It has been reported that treatment with anti-CTLA-4 resulted in rejection of two fibrosarcomas when begun 1–2 wk after inoculation but that late-stage tumors (7–10 wk) were resistant to treatment (26). This loss of effectiveness was accompanied by a loss of in vitro antitumor responses, suggestive of deletion or inactivation of T cells. It has also been shown that growth of a B cell lymphoma engineered to express influenza hemagglutinin results in the progressive inactivation of adoptively transferred T cells bearing hemagglutinin-specific TCRs (27). In this system, administration of anti-CTLA-4 greatly enhanced T cell priming if begun before responses were totally lost but could not reverse tolerance once established (27a). The basis for loss of responsiveness of more established B16-BL6 tumors to the combined treatment regimen remains to be established.

Our results demonstrate that both the therapeutic effect and the subsequent depigmentation obtained with the combination treatment required $CD8^+$ and $NK1.1^+$ cells but was independent of $CD4^+$ T cells. The involvement of $NK1.1^+$ cells in prophylaxis induced by B16/GM-CSF vaccines has been previously noted, especially when MHC Class I^{lo} or I^- cells were used (28, 29). It is therefore not surprising that eradication of B16-BL6 in our model might require $NK1.1^+$ cells. An important contribution of the $NK1.1^+$ cells may be to lyse cells in the vaccine, thereby enhancing antigen uptake by host APCs recruited to the site of vaccination by GM-CSF in the vaccine.

Our previous studies have revealed that tumor rejection after anti-CTLA-4 treatment, given alone in the case of immunogenic tumors or together with a GM-CSF–transduced vaccine for a poorly immunogenic mammary carcinoma, required both $CD4^+$ and $CD8^+$ cells (14, 17). This result could be interpreted as indicative of a requirement for $CD4^+$ T cell help for the effective induction of $CD8^+$ CTLs. However, several observations have suggested a role for $CD4^+$ T cells in antitumor responses beyond provision of help for CTLs. Depletion of $CD4^+$ or $CD8^+$ T cells after immunization but before tumor challenge abrogates the ability of irradiated GM-CSF–producing B16 cells to induce protective immunity (20). $CD4^+$, but not $CD8^+$, T cells were required for the induction of immunity with a GM-CSF–expressing, class I MHC–negative tumor cell vaccine (28). Finally, an extensive analysis using a variety of knockout mice as hosts has shown that $CD4^+$ T cells were absolutely required for the induction of protective immunity using GM-CSF–expressing B16 cells but that absence of $CD8^+$ T cells resulted in only a partial loss of effectiveness (22). This, together with the observation that cytokines elaborated by $CD4^+$ T cells resulted in the recruitment and activation of eosinophiles and macrophages, suggested an additional role for $CD4^+$ T cells in orchestrating $CD8^+$ T cell–independent protective mechanisms when GM-CSF–expressing B16 cell vaccine is used in the setting of prophylaxis.

In the therapeutic setting, our finding that $CD4^+$ T cells are dispensable for obtaining tumor rejection suggests that the combination treatment in this system can allow for direct induction of $CD8^+$ T cell responses, in agreement with what has recently been reported for antiparasite responses (30). One contributing factor might be a high dose and persistence of antigen due to the use of three doses of tumor cell vaccine. It is also possible that CTLA-4 blockade lowers the threshold of stimulation or costimulation that is required for activation of naive T cells. It has recently been shown that a very important mechanism of $CD4^+$ T cell help for the generation of CTLs is an enhancement of antigen presentation and costimulatory activity of dendritic cells as a consequence of engagement of CD40 on the dendritic cell by CD40L on activated $CD4^+$ cells (31–33). It is possible that CTLA-4 blockade lowers the threshold of signals needed for $CD8^+$ T cell activation to a level that can be provided by GM-CSF–stimulated dendritic cells in the absence of "licensing" by activated $CD4^+$ T cells.

After eradication of B16-BL6 tumors, 56% of the surviving mice developed depigmentation starting at the sites of vaccination and challenge and spreading to distant sites. Loss of coat color indicated that systemic and progressive autoimmunity had developed toward pigment-bearing cells. For human melanoma patients, a good correlation between autoimmune depigmentation and improved clinical response has been documented (34, 35). Melanoma-associated hypopigmentation closely resembles vitiligo, an autoimmune phenomenon that possibly involves antibody and T cell responses against melanocyte antigens (36, 37). Genes encoding proteins associated with pigment synthesis or with melanosomes have been cloned and characterized as targets for CTLs in human melanoma patients (1, 2). Reinfusion of autologous tumor-infiltrating lymphocytes specifically recognizing gp100/Pmel-17 or tyrosinase led to tumor regressions in some cases, although the value of targeting such antigens is unclear from such clinical studies because the adoptive transfers were performed in the presence of high-dose systemic IL-2 (38). Apparently, T cell tolerance against these melanocyte antigens can be broken to induce antitumor reactivity. Currently, several approaches (peptide or genetic vaccination), to (re-)direct melanocyte-reactive CTLs against melanoma are clinically evaluated. The consequences of breaking tolerance to pigment antigens are largely unknown and could be studied in an appropriate murine model.

Two murine melanocyte antigens (TRP2 and Pmel-17/gp100) have been found to serve as CTL antigens in the immune response to B16 tumors (6, 39). T cell tolerance toward Pmel-17/gp100 could only be broken by using xenogeneic human gp100. No autoimmune depigmentation was reported after vaccination with peptide or recombinant vaccinia virus or after adoptive transfer of specific CTL clones. In addition to CTLs, potent antibody responses were generated against gp75/TRP1 by vaccinating naive mice with recombinant human gp75, hgp75 DNA, human melanoma cells expressing gp75, or recombinant vaccinia virus expressing human gp75 but not using murine gp75 formulations (40–42). Apparently, B cell tolerance toward gp75

was broken by using xenogeneic antigen. Follow-up studies demonstrated that tumor protection required $CD4^+$ and $NK1.1^+$ cells but not $CD8^+$ cells, whereas depigmentation developed in $CD4^{-/-}$ and $FcR\gamma^{-/-}$ mice in the absence of tumor protection, suggesting that the phenomena are caused by different mechanisms (43). It should be noted that gp75/TRP1 is the most abundant protein in melanocytes and some melanomas, and it can be detected on the cell surface (in contrast to the other melanocyte antigens), which could explain the finding of autoimmune depigmentation associated with anti-gp75 antibodies.

In our system, tumor rejection induced by a combination of the BL6/GM-CSF vaccine and CTLA-4 blockade was followed by depigmentation, which can occur in the absence of $CD4^+$ T cells. Depigmentation was not observed in any of the small number of mice whose tumors were rejected after treatment with the vaccine alone, nor was depigmentation noted in previous studies of GM-CSF/B16 vaccines used for prophylaxis (20, 22). It seems likely that depigmentation occurs in our system because the GM-CSF vaccine, when enhanced by CTLA-4 blockade, can elicit CTLs directed to normal melanocyte antigens expressed by the tumor cells, and the same cells responsible for tumor rejection also mediate autoimmune destruction of normal melanocytes. However, it remains possible that antibodies to gp75 or other antigens have some role in depigmentation in intact mice.

In our view, there are at least two nonexclusive explanations for our observation that anti–CTLA-4 antibodies synergize with BL6/GM-CSF vaccine to induce rejection and autoimmunity: (a) CTLA-4 blockade greatly increases the burst size of T cells responding to the GM-CSF vaccine, thus enhancing the mobilization of effector cells, and (b) CTLA-4 blockade lowers the threshold for T cell activation, thereby allowing the recruitment and activation of low-affinity autoreactive T cells that might have escaped central tolerance induction. In either case, autoreactive CTLs involved in tumor rejection could find targets in melanocytes exposed through local inflammation or skin destruction. Although it is an unwanted side effect of treatment, depigmentation or vitiligo is considered to be an acceptable risk for the treatment of melanoma in clinical situations. To our knowledge, this report is the first describing T cell–dependent depigmentation after successful treatment of murine melanoma. Rejection of B16-BL6 through CTLA-4 blockade plus GM-CSF–producing vaccines could serve as a model to study the relationship between tumor immunity and autoimmunity in a setting relevant to the treatment of human cancer.

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Immunotherapy of Primary Prostate Cancer in a Transgenic Model Using a Combination of CTLA-4 Blockade and Tumor Cell Vaccine

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Abstract:

We have previously shown that antibodies to CTLA-4, an inhibitory receptor on T cells, can be effective at inducing regression of transplantable murine tumors. In this study, we demonstrate that an immune response against primary prostate tumors in transgenic (TRAMP) mice can be elicited using a strategy that combines CTLA-4 blockade and an irradiated tumor cell vaccine. Treatment of TRAMP mice at 14 weeks of age resulted in a significant reduction in tumor incidence (15% vs control, 75%) when assessed 8 weeks after vaccination. Histopathological analysis revealed that treated mice had a lower tumor grade. TRAMP mice treated with anti-CTLA-4 and a vaccine genetically modified to express GM-CSF exhibited significant accumulation of inflammatory cells in the interductal spaces. Vaccination of non-transgenic mice with the same cell-based vaccine and anti-CTLA-4 resulted in prostatitis, indicating that the immune response was in part directed against normal prostate antigens. These findings demonstrate that this combinatorial vaccine can elicit a potent anti-tumor response directed, in part, against normal prostate antigens and suggest potential of this approach for treatment of prostate cancer in man.

Introduction:

Recent advances in our understanding of the mechanisms regulating T cell activation have allowed the development of better strategies for the immunotherapy of cancer. It has become clear, for example, that full activation of naïve T cells requires not only stimulation of the antigen receptor by peptide/MHC complexes, but also costimulatory signals mediated by engagement of CD28 by B7 (CD80 or CD86) [1]. B7 expression is limited to "professional" antigen presenting cells such as dendritic cells, macrophages, and activated lymphocytes [1]. One of the reasons for the poor immunogenicity of tumors may be their lack of expression of these costimulatory ligands [2,3]. Induction of B7 expression on murine tumor cells by genetic modification has been shown in several systems to greatly enhance the effectiveness of tumor cell vaccines in providing protection against tumor challenge. However, B7+ tumor cells have not been found to be particularly effective in treating established tumors (reviewed in [4,5]). Other strategies in tumor immunotherapy rely, at least in part, on enhancing costimulation. These include the use of tumor cells transduced to express granulocyte-macrophage colony-stimulating factor (GM-CSF) to enhance cross-priming of T cells by professional antigen presenting cells (APCs) [6], dendritic cells pulsed with peptides [7,8] or RNA [9] to provide immunization in the context of a potent APC, anti-CD40 antibodies to enhance expression of costimulatory ligands on APCs [10-12], and IL-2 to bypass the need for costimulation [13-15].

More recently, costimulation has been shown to be more complex than previously thought; CTLA-4 is a second T cell counter-receptor for B7 [16] that plays a critical role in attenuating T cell responses. CTLA-4 engagement may inhibit the initiation of T cell responses by raising the threshold of signals needed for full activation, or may also play a role in terminating ongoing T cell responses [17,18]. Anti-CTLA-4 antibodies which block CTLA-4/B7 interactions

enhance *in vivo* T cell responses to peptides, superantigens, and parasites, and can exacerbate experimental autoimmune encephalomyelitis (for review, see [17]). Administration of anti-CTLA-4 antibodies is sufficient to induce the rejection of newly implanted and in some cases well established tumors in several transplantable murine tumor systems [19-22]. The effectiveness of CTLA-4 blockade in these systems appears to be dependent on the inherent immunogenicity of the tumor. While CTLA-4 blockade by itself is not effective in the treatment of poorly immunogenic transplantable tumors such as the mammary carcinoma SM1 [23] or the melanoma B16 [24], eradication of these tumors can be achieved when anti-CTLA-4 is administered together with an irradiated tumor cell vaccine expressing GM-CSF. In the case of the B16 melanoma, tumor rejection is regularly accompanied by a progressive depigmentation resembling the vitiligo that accompanies immunotherapy in many human melanoma patients [25-28]. This result suggests that in mice, as in man, the anti-melanoma response is at least in part directed to normal melanocyte-specific antigens [24,25].

In contrast to the considerable literature documenting immunological responses to melanoma in humans and in mouse models, there is a paucity of data concerning immunological responses to prostate tumors. We have shown that CTLA-4 blockade is sufficient to obtain partial or complete regression of subcutaneous implants of tumor cell lines [29] derived from the **TR**ansgenic **A**denocarcinoma of **M**ouse **P**rostate (TRAMP) mice in syngeneic, non-transgenic C57Bl/6 male mice [20]. In the current study, we examined the potential of CTLA-4 blockade in the treatment of primary cancer in TRAMP mice. We found that CTLA-4 blockade in combination with irradiated tumor cell vaccines was effective in reducing tumor incidence and severity of prostatic lesions. We also noted significant accumulation of inflammatory cells in the prostates of some vaccinated TRAMP mice. Finally, we show that the anti-tumor response

is directed in part to antigens expressed by normal prostate, since immunization of non-transgenic mice with GM-CSF-expressing tumor cell vaccines under conditions of CTLA-4 blockade results in marked prostatitis. This work demonstrates for the first time the effectiveness of this immunotherapeutic regimen in primary cancer, and indicates that prostatic tumors express tissue-specific antigens that may provide targets for immunotherapy.

Results:

Given the potency of CTLA-4 blockade combined with cell-based vaccines in poorly immunogenic transplantable tumor models, we examined the effectiveness of this strategy in the treatment of primary prostatic cancer in TRAMP mice [30]. In these mice, SV40 T antigen transgene expression is under the transcriptional control of the rat probasin promoter that directs expression of the oncogene to prostatic epithelium in an androgen-regulated manner. Pathogenesis of neoplasia in TRAMP mice mirrors that in man. When transgene expression begins at puberty, male TRAMP mice develop hyperplasia (5-8 weeks of age), frank neoplasia (8-12 weeks) and eventually invasive adenocarcinoma with metastasis to the lungs, lymph nodes and bone (15-20 weeks) [31]. Using early-passage cell lines (TRAMP-C1 and TRAMP-C2) derived from an advanced tumor in a TRAMP mouse, we previously demonstrated that CTLA-4 blockade is sufficient to obtain partial or complete regression of subcutaneous implants of these lines in syngeneic, non-transgenic C57Bl/6 male mice [20]. These findings, together with those using poorly immunogenic transplantable tumors [23,24], led us to treat primary prostate cancer in the TRAMP model using anti-CTLA-4 itself and in combination with cell-based vaccines.

Reduction of primary tumor incidence in TRAMP mice following treatment with cell-based vaccines and anti-CTLA-4

Male TRAMP mice were vaccinated with a combination of irradiated TRAMP-C1 and TRAMP-C2 (TRAMP-C1/C2) or TRAMP-C1/C2 transduced to express the murine *gm-csf* gene (GMTRAMP-C1/C2) at about 3.5 months of age. Antibody treatment was begun 7 days after vaccination. To obtain an early indication of the effectiveness of the treatments, four mice from each group

were euthanized three weeks after commencement of treatment and examined for tumor incidence at gross necropsy and at the microscopic level following microdissection of the prostatic lobes. While there were no significant differences in mean animal or urogenital tract weight between the treatment groups, there was a striking difference in tumor incidence. Irrespective of vaccine, 11 of 12 mice (92%) in the treatment groups receiving control antibody had detectable tumor. In contrast, only 3 of 12 (25%) mice receiving anti-CTLA-4 had detectable tumor.

At three weeks after treatment, the tumors in the control antibody-treated mice were sufficiently large to warrant concern about survival of the remaining 150 mice. Therefore, to allow assessment of tumor incidence and tumor grade, the remaining 25 mice in each group were euthanized 5 weeks later (or eight weeks after treatment), and the following criteria assessed at gross necropsy and microdissection of the prostatic complex: animal weight, prostate weight, tumor incidence, and histopathology of prostatic disease. Similar to the analysis at 3 weeks after treatment, there was no significant difference in animal weight or prostate weight between any of the treatment groups. However, there were significant differences in tumor incidence (figure 1A). A significantly lower tumor incidence was observed in mice treated with anti-CTLA-4 and either the TRAMP-C1/C2 vaccine (43%, P=.05) or the GMTRAMP-C1/C2 vaccine (33%, P=.009) than in mice treated with control antibody alone (69%). Treatment with anti-CTLA-4 alone had no significant effect on tumor incidence (64%), nor was there significant reduction in tumor incidence in mice receiving the control antibody treatment and either vaccine (55%-TRAMP-C1/C2 and 75%-GMTRAMPC1/C2). Thus, neither CTLA-4 blockade nor vaccination alone was effective at treating primary tumors in TRAMP mice. However, the combination of anti-CTLA-4 and either vaccine had a synergistic effect on tumor incidence. The expression of GM-CSF by the vaccine may potentiate the anti-tumor response since the tumor incidence was

slightly lower in mice vaccinated with GMTRAMP-C1/C2 (33% anti-CTLA-4+GMTRAMP-C1/C2 versus 43%-anti-CTLA-4+TRAMP-C1/C2).

Because each group contained mice from litters with birthdates two weeks apart, tumor incidence was reassessed as a function of age at the initiation of treatment. As shown in figure 1B for mice vaccinated with GMTRAMP-C1/C2, there was significant reduction in tumor incidence in the mice treated at 14 weeks of age (p=.003), but not in the group treated at 16 weeks of age (p=.1). This suggests that the stage of tumor development at the time of immunotherapy of TRAMP mice influenced the efficacy of treatment. Tumor incidence in mice treated with TRAMP-C1/C2 and anti-CTLA-4 was equivalent at either age of treatment and was not significantly different from control mice.

Reduction of tumor grade in TRAMP mice treated with combination immunotherapy

To assess the severity of prostate lesions in TRAMP mice, the individual lobes of the prostate were prepared for routine histopathological analysis. A scoring scale was used to evaluate the extent of transformation or tumor grade observed in the prostates of TRAMP mice (as described in Methods, [31]). The peak histological score for the ventral, dorsal or lateral prostate lobes was determined for each animal and the average for the treatment group calculated as a mean peak score. As shown in figure 2, there was a significant reduction in the severity of lesions in mice treated with anti-CTLA-4 and either vaccine. Specifically, TRAMP mice treated with TRAMP-C1/C2 and anti-CTLA-4 had a significantly lower score (mean peak score 4.6) than control Ig-treated mice (mean peak score 5.5, p=.03). Even more striking was the finding that mice treated with GMTRAMP-C1/C2 and anti-CTLA-4 had a significantly lower tumor grade (mean peak score 3.9) than all three control groups: control Ig/no vaccine (p=.0009), control Ig/GMTRAMP-C1/C2 (mean peak score 5.5, p=.0002),

and anti-CTLA-4 treatment alone (mean peak score 4.8, p=.04). Treatment with either vaccine without CTLA-4 blockade or CTLA-4 blockade alone had no significant effect on tumor grade. These findings demonstrate that in addition to reducing the incidence of primary tumors at 8 weeks after treatment, vaccination with a tumor cell-based vaccine in combination with CTLA-4 blockade reduces the severity of prostatic lesions in TRAMP mice.

As was performed for analysis of tumor incidence, the histological data was reanalyzed for tumor grade as a function of age at time of treatment. Similar to tumor incidence, the highest statistical significance resided in mice treated at 14 weeks of age. Mice treated with GMTRAMP-C1/C2 and anti-CTLA-4 (mean peak score=3.5) had a lower tumor grade than mice treated with GMTRAMP-C1/C2 and control Ig (mean peak score=5.3, p=.0002) and mice treated with control Ig alone (mean peak score=4.7, p=.0002). Interestingly, when treated at 16 weeks of age, TRAMP mice receiving the GMTRAMP-C1/C2 vaccine and anti-CTLA-4 (mean peak score=4.5) only had a slightly lower mean peak score than mice treated with GMTRAMP-C1/C2 and control Ig (mean peak score=5.6, p=.03).

Perhaps the most striking histological feature of these analyses was observed in mice treated with GMTRAMP-C1/C2 and anti-CTLA-4, where there was an accumulation of inflammatory cells in the interductal spaces (figure 3c,d). In these mice, inflammatory cells were closely associated with the vasculature found in the stroma. In contrast, there was no detectable accumulation of inflammatory cells in any of the control Ig-treated mice (figure 3b). In TRAMP mice treated with a GM-CSF-expressing vaccine alone, there were occasional areas where inflammatory cells were detected but these sites were not as extensive as those observed in mice also treated with anti-CTLA-4 (data not shown). The morphological features of the infiltrating cells suggested that the perivascular inflammation was comprised of myeloid as well as lymphoid cells. The identity of these infiltrating cells will be established by immunhistochemical analyses.

Induction of Prostatitis in Non-Transgenic mice by Vaccination and CTLA-4 Blockade

The reduction in incidence and severity of tumors together with the inflammatory infiltrates of the prostate in the TRAMP mice eight weeks after immunization were indicative of a potent immune response. The fact that tumorigenesis in these mice is driven by prostate-specific expression of SV40 Tag raised the possibility that the anti-tumor response was directed against epitopes derived from this viral oncogene. We considered this to be unlikely since TAg expression could not be detected in the vaccine tumor cells by RT-PCR [20], nor were the tumor cells lysed by CTL reactive against H-2^b-restricted epitopes of Tag (personal communication, S. Tevethian and L. Mylin, Pennsylvania State University). To determine whether the immune response elicited by the therapeutic regimen was limited to oncogene-encoded antigens, non-transgenic C57/BL6 mice were vaccinated and the prostates examined for evidence of inflammation 28 days later. Examples of tissue sections from vaccinated mice are shown in figure 4. There was no evidence of significant inflammation or tissue damage in the dorsolateral or ventral lobes of the prostates of mice vaccinated with the GMTRAMP-C1/C2 vaccine only. However, there was extensive mononuclear cell infiltration and destruction of glandular epithelium of the male reproductive tract including the dorsolateral prostate in mice vaccinated with GMTRAMP-C1/C2 and treated with anti-CTLA-4. These results demonstrate that the response elicited by the vaccination regimen is directed in part to antigens expressed by normal prostate cells.

Discussion:

The data presented in this report extend our previous findings and demonstrate that CTLA-4 blockade can be combined with cytokine-expressing, cell-based vaccines to produce an effective treatment regimen for the treatment of primary tumors. TRAMP mice develop primary, autochthonous tumors with a pathoge-+nesis similar to prostate cancer in man. Using this model, we demonstrated that vaccination with TRAMP-derived TRAMP-C cells combined with anti-CTLA-4 treatment resulted in a reduction in both tumor incidence and the tumor grade. Histological analyses also revealed significant accumulation of inflammatory cells in the interductal spaces of TRAMP mice treated with GM-CSF-expressing vaccine and anti-CTLA-4. This immunization regimen also induced prostatitis in non-transgenic mice, indicating that the targets of this vaccination approach include self antigens expressed in normal as well as neoplastic prostatic tissues.

The reduction of both tumor incidence as well as histological tumor grade indicates that the combination of a cell-based vaccine together with anti-CTLA-4 was sufficient to slow the progression of primary prostatic tumors. Because the TAg transgene is under the transcriptional control of an androgen-regulated promoter and is therefore constitutively active in prostatic lumenal epithelial cells of transgenic mice after sexual maturation, over time, a transformed phenotype will be observed in all prostatic epithelium. It is not surprising that the immune system is unable to completely eliminate tumors in this aggressive model, but it is remarkable that an anti-tumor immune response can have a significant impact on disease progression in a situation where an entire organ is undergoing transformation. While the development and application of murine models with temporally regulated tissue specific transgene expression are being explored as less virulent models of human tumorigenesis, the ability of a cell-based vaccine in combination with CTLA-4-blockade to significantly reduce tumor incidence and burden in the aggressive

TRAMP model underscores the remarkable efficacy of this immunotherapeutic approach.

Our data in this primary tumor model indicate a synergy between CTLA-4 blockade and a tumor cell-based vaccine. TRAMP mice treated with either the vaccine or antibody alone had no reduction in tumor incidence or tumor grade whereas the combination of both resulted in a significant reduction in both criteria. This suggests that an additional source of antigen from the cell-based vaccine contributes to T cell priming, which is enhanced by blockade of CTLA-4/B7 interactions. The fact that tumor incidence and tumor grade were lower in mice that received the GMTRAMP-C1/C2 vaccine than those receiving the TRAMP-C1/C2 vaccine suggest that the effect is enhanced by the recruitment and activation of APCs by GM-CSF expression.

Somewhat surprisingly, the effectiveness of treatment was heavily influenced by the age of the animal at the time of treatment. Mice treated with anti-CTLA-4 and the TRAMP-C1/C2 vaccine at 14 weeks of age had a significantly lower tumor incidence and tumor grade. In contrast, treatment at 16 weeks of age resulted in only a slight reduction in tumor grade but no reduction in tumor incidence. The basis for this age dependence is not clear. The accessibility of the tumor to the immune system may change with the progression of neoplasia due to alterations in vasculature or intratumoral pressure. Tumor growth during this time period may begin to exceed the ability of the immune system to have a significant impact on controlling tumor growth. However, at 14 and 16 weeks of age, there does not appear to be any histopathological differences that might suggest that the antigenic profile might differ between these two ages.

Vaccination of non-transgenic mice with the same therapeutic strategy demostrated to be effective for treatment of TRAMP mice led to autoimmune prostatitis and destruction of some prostatic epithelium. This finding suggests that the vaccination approach is capable of inducing an autoimmune response against normal prostate antigens. We have observed development of

autoimmune depigmentation following rejection of a pigmented melanoma using a combination of CTLA-4 blockade and melanoma cells expressing GM-CSF [24]. This is similar to the vitiligo that has been observed in patients showing clinical responses to immunotherapy of melanoma [25]. These results add support to the idea that effective tumor immunity is, in fact, closely tied to autoimmunity. Rather than being viewed as a troublesome side effect of tumor immunotherapy, an emerging concept is that intentional induction of autoimmunity to defined tissue-specific antigens may be a practical strategy for generation of effective anti-tumor responses [28,32]. The findings presented in this report support this approach for immunological treatment of tumors arising from non-essential tissues.

The work presented here demonstrates the successful treatment of primary tumors using a cell-based vaccine combined with administration of an anti-CTLA-4 antibody. We are currently examining the effectiveness of CTLA-4 blockade in combination with more conventional therapies such as androgen ablation or chemotherapy that might induce sufficient tumor cell death to achieve some priming of tumor-reactive T cells. We will also attempt to identify, by expression cloning, the prostate antigens that serve as targets for the immune responses elicited by tumor cell vaccines in combination with CTLA-4 blockade. Identification of these targets might allow immunization against defined antigens of known distribution and provide a more focused immune response. Recent data provide compelling support for the therapeutic potential of the blockade of inhibitory signal of T cell activation mediated by CTLA-4 as a strategy for enhancing immunological responses to tumors.

Methods:

Mice: All animal procedures were performed according NIH guidelines under protocols approved by the University of California Animal Care and Use Committee. TRAMP mice were bred within our colony on a pure C57BL/6 background. For these experiments, TRAMP mice were backcrossed one time with FVB/N mice and screened for the presence of the transgene by PCR as previously described [30].

Mice were vaccinated subcutaneously with 1 x 10^6 cells each of irradiated (12,000 rads) TRAMP-C1 and TRAMP-C2 or their GM-CSF-transduced derivatives, GMTRAMP-C1/C2. To maximize antigenic challenge, this treatment was repeated two additional times, three days apart. Seven days after the initiation of vaccination, mice were injected intraperitoneally with 100 μg of anti-CTLA-4 (clone 9H10, purified over protein G as previously described [33]) or with purified hamster IgG (Jackson Immunoresearch Corp., West Grove, PA). Additional doses of antibody were administered 3 and 6 days after the first treatment. Mice were followed for morbidity and were euthanized when tumor burden exceeded approximately 50 mm in diameter or animal respiration was strained. Mice were euthanized at the indicated age and the prostatic complex microdissected under a stereomicroscope. Tumor incidence was initially assessed at necropsy and confirmed by histopathologic examination.

Histopathological Analyses: The prostatic complex was microdissected into the individual lobes and fixed in 10% neutral buffered formalin. Tissues were processed and stained with hematoxylin and eosin for routine histopathologic analyses. TRAMP tissues were examined by light microscopy and scored using the following criteria [31]: Normal epithelium was assigned a score of 1.0; early

signs of prostatic intraepithelial neoplasia (PIN) with tufting of the epithelium and increased nucleus:cytoplasm ratio were scored as 2.0; more advanced PIN with noted cribiform structures and increase in mitotic and/or apoptotic figures was scored as 3.0; the loss of interductal spaces and the invasion of basement membranes by neoplastic epithelium was scored as 4.0; total loss of ductal lumens with evidence of adenocarcinoma was scored as 5.0; and sheets of anaplastic tumor cells were scored as 6.0. To generate a score for each animal, the maximum histologic score for the ventral, dorsal or lateral prostate lobes was used to calculate a mean for the treatment group. The predominant peak score for all TRAMP animals was 4.0 with few histologic scores below 3.0.

Cell Culture: TRAMP-C cells are early passage (10-15 passages *in vitro)*, non-clonal epithelioid tumor cells independently derived from a TRAMP mouse [29]. Cells were propagated in culture using DMEM (Biowhittaker, Walkersville, MD) supplemented to a final concentration of 5 % fetal calf serum (Biowhittaker), 5 % Nu-Serum (Collaborative Biomedical Products, Bedford, MA), 5 μg/ml insulin (Sigma Chemical, St. Louis, MO), and 0.01 μM dihydrotestosterone. To obtain GM-CSF-expressing lines, cells were infected with a retrovirus containing the mouse *gm-csf* gene driven by the Maloney murine leukemia virus LTR, using the ψCRIP producer line (gift from Somatix, Inc, Alameda, CA). Retrovirus-containing supernatants were added to TRAMP-C cultures and incubated overnight in the presence of 8 μg/ml polybrene (Sigma). GM-CSF production was assayed by ELISA (Pharmingen, San Diego, CA). Both GMTRAMP-C1 and GMTRAMP-C2 secreted GM-CSF at 150-200 ng/ml/$1x10^6$ cells/24 hours. Cells used for injection were released from tissue culture dishes with trypsin (BioWhittaker) and washed three times in Hank's balanced salt solution (BioWhittaker). Cells were resuspended at a density of $1x10^7$ cells/ml, irradiated with 12,000 rads using a cesium-source irradiator and injected subcutaneously in a volume of 0.1 ml.

Figure Legends:

Figure 1: TRAMP mice treated with TRAMP-C cells and anti-CTLA-4 have a lower tumor incidence than control-treated animals. Mice were vaccinated at 14-16 weeks of age with the indicated irradiated cell vaccine and injected i.p. with either hamster anti-CTLA-4 or a control hamster IgG fraction (ctrl Ig). (A) As indicated, mice treated with a TRAMP-C vaccine and anti-CTLA-4 had a significantly lower tumor incidence (43%) than ctrl Ig-treated mice (63%, P=0.05). Mice treated with GMTRAMP-C1/C2 and anti-CTLA-4 had an even lower tumor incidence (33%, P=.01) than sham-treated mice; there was no significant difference between mice treated with the two different vaccines. (B) Reanalysis of data presented in figure (A) for tumor incidence as a function of age at the time of treatment revealed that the significant reduction in tumor incidence was from the mice treated at 14 weeks of age. In contrast, there was no significant reduction in tumor incidence in mice treated at 16 weeks of age.

Figure 2: TRAMP mice treated with TRAMP-C vaccines and anti-CTLA-4 exhibit a reduction in the severity of prostatic lesions. At necropsy, prostatic tissues were microdissected, fixed and prepared for routine histopathological analyses. Tissues were scored for the progression of disease as described in Methods presented as the mean peak score +/- standard deviation. (A) In mice treated with unmodified TRAMP-C vaccines and anti-CTLA-4 (mean peak score 4.6), there was a significant reduction in severity of lesions compared to sham-treated mice (ctrl Ig/no vaccine, mean peak score 5.5). In mice treated with both the GMTRAMP-C1/C2 vaccine and anti-CTLA-4 (mean peak score 3.9), there was a significant reduction in histological severity of disease compared to sham-treated or both single-treatment groups. (B) Reanalysis of data in (A) demonstrates that similar to tumor incidence, TRAMP mice treated with

GMTRAMP-C1/C2 and anti-CTLA-4 had a reduction in tumor grade when treated at 14 weeks of age but not when treated at 16 weeks.

Figure 3: Treatment of TRAMP mice with GMTRAMP-C vaccines and anti-CTLA-4 results in accumulation of inflammatory infiltrates in the prostatic acinar structures. Tissues were microdissected following treatment and prepared for histopathological analyses as described in Methods. In contrast to non-transgenic dorsolateral prostate (A), severe transformation of TRAMP prostatic tissues was observed (B-D). Varying degrees of histological severity were observed in ctrl Ig-treated TRAMP mice, ranging from advanced cribiform structures with occlusion of ductal structures to anaplastic adenocarcinoma (B). Less severe disease was observed in mice treated with GMTRAMP-C1/C2 and anti-CTLA-4 (C,D), with significant accumulation of inflammatory cells (arrows) in the interductal spaces, closely associated with the vasculature.

Figure 4: Generation of prostatits in wild-type mice treated with a GMTRAMP-C vaccine and anti-CTLA-4. 12 week-old, non-transgenic C57BL/6 male mice were sensitized with GMTRAMP-C1/C2 vaccines and given anti-CTLA-4 as described in Methods. The prostatic complex was microdissected and processed for histological analysis. In contrast to dorsolateral prostate of mice treated with GMTRAMP-C1/C2 and (A), significant inflammatory infiltrates (arrows) were observed in vaccinated mice, 4 weeks after treatment (B). In some areas, tissue damage included destruction of acinar structures (arrowheads).

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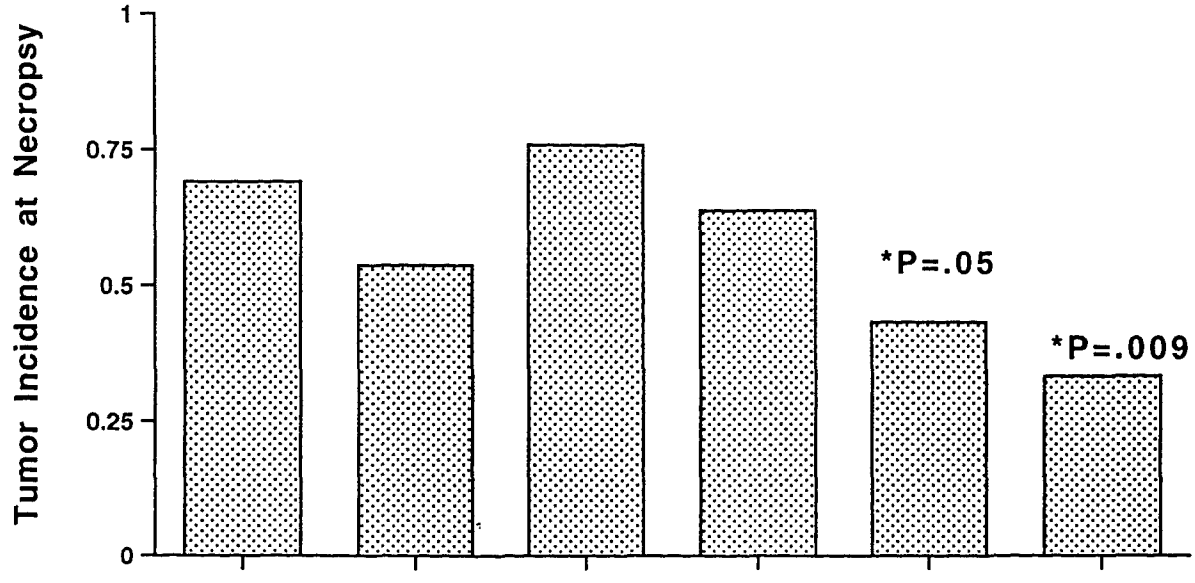
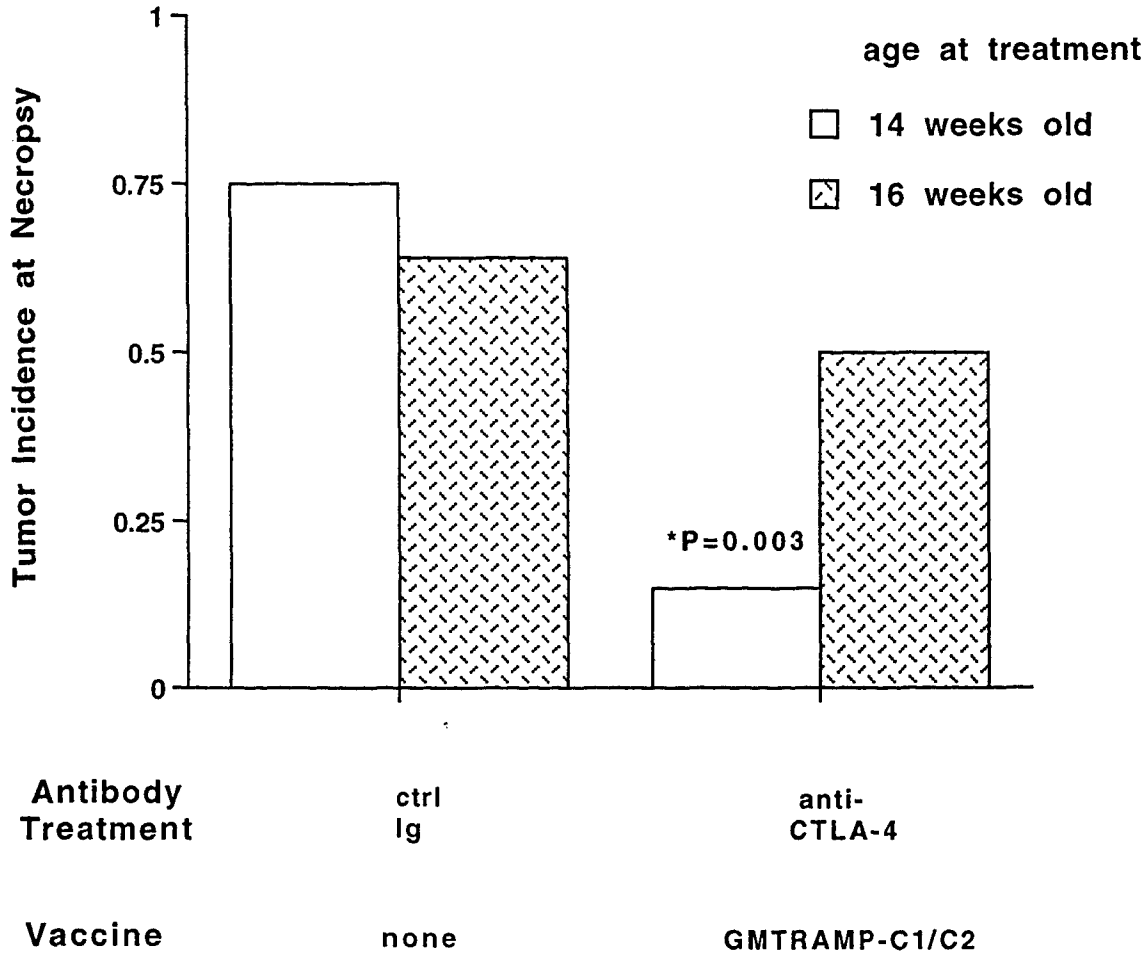
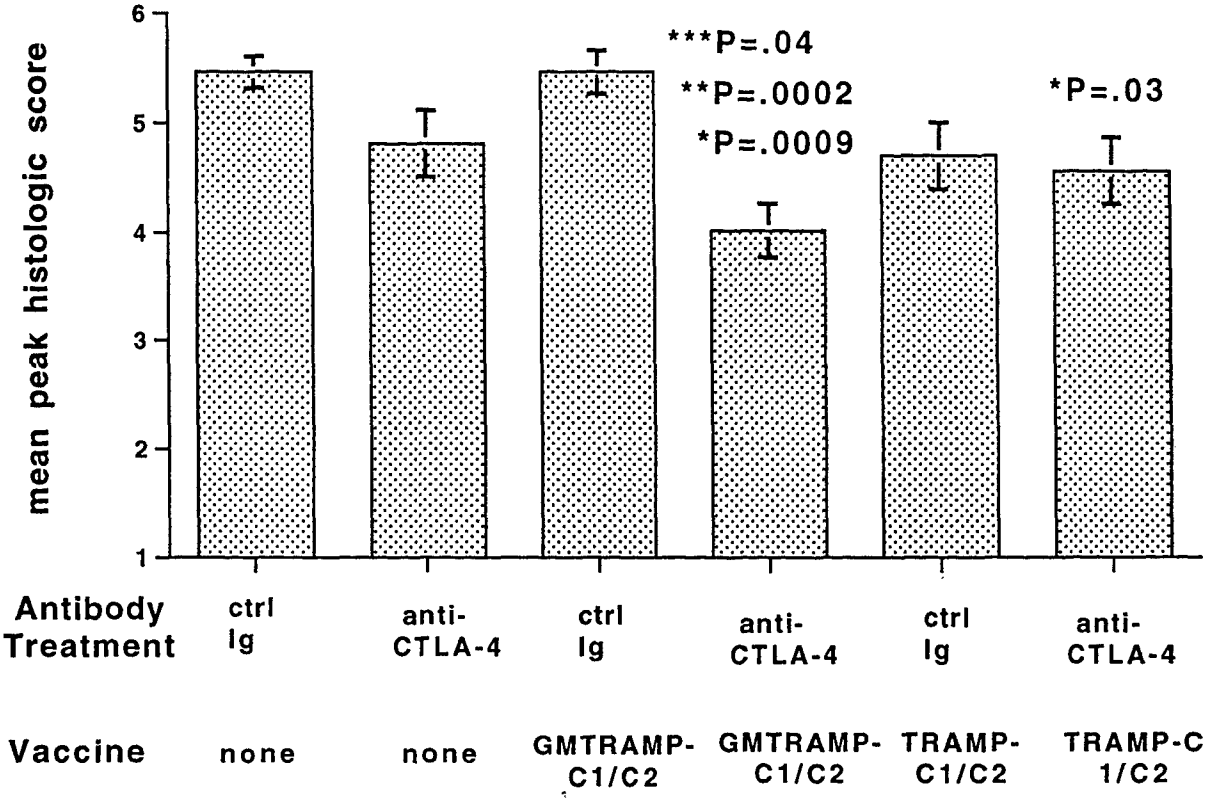


Hurwitz *et al*, figure 1A



(*=χ2 vs. ctrl Ig/no vaccine, treated at same age-14 weeks)

Hurwitz *et al*, figure 1B



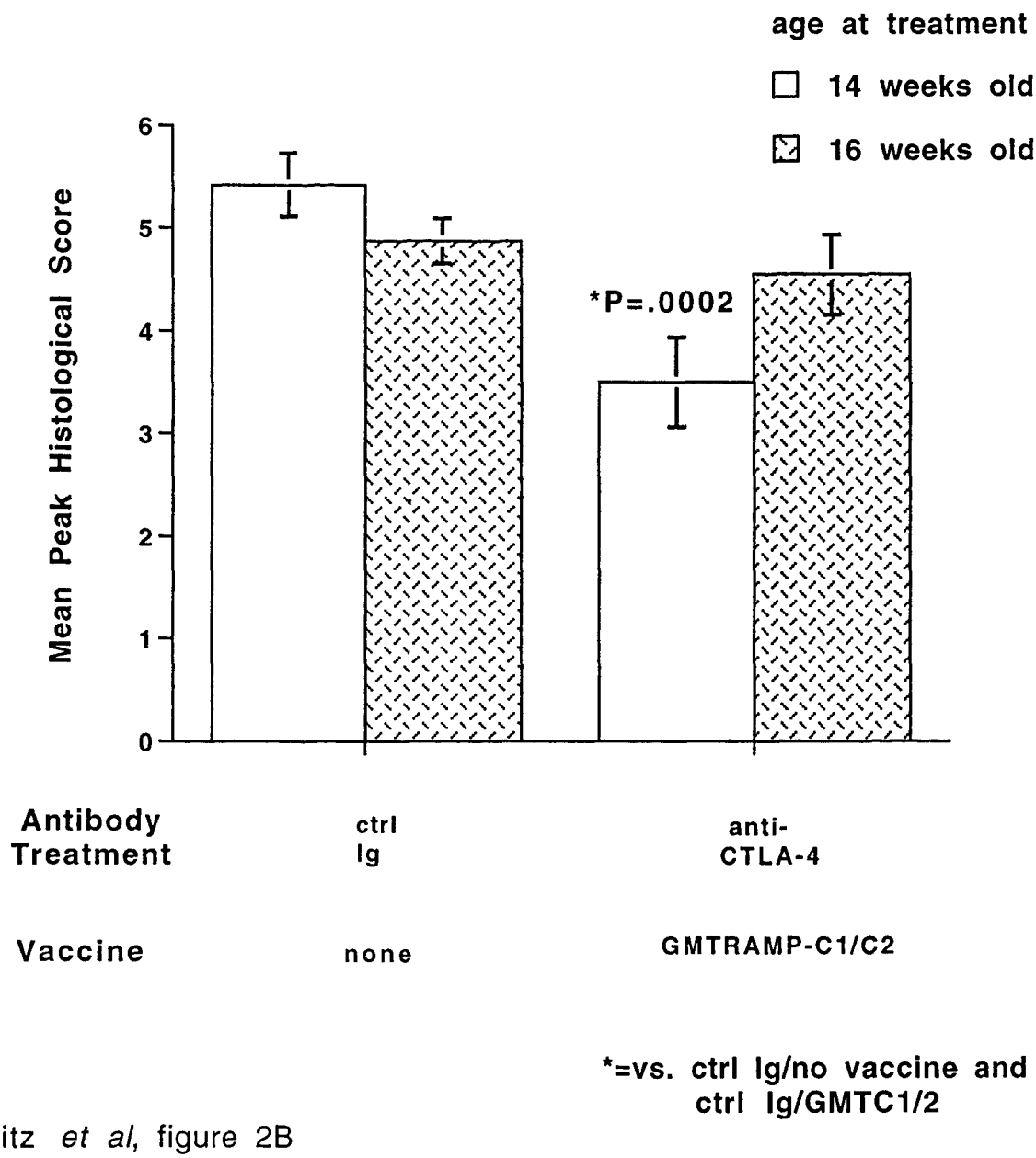
*=vs. ctrl Ig/no vaccine

**=vs. ctrl Ig/GMTRAMP-C1/C2

***=vs. anti-CTLA-4/no vaccine

Statistically Significant Using Fisher,Scheffe,Bonferroni/Dunn Tests

Hurwitz *et al*, figure 2A



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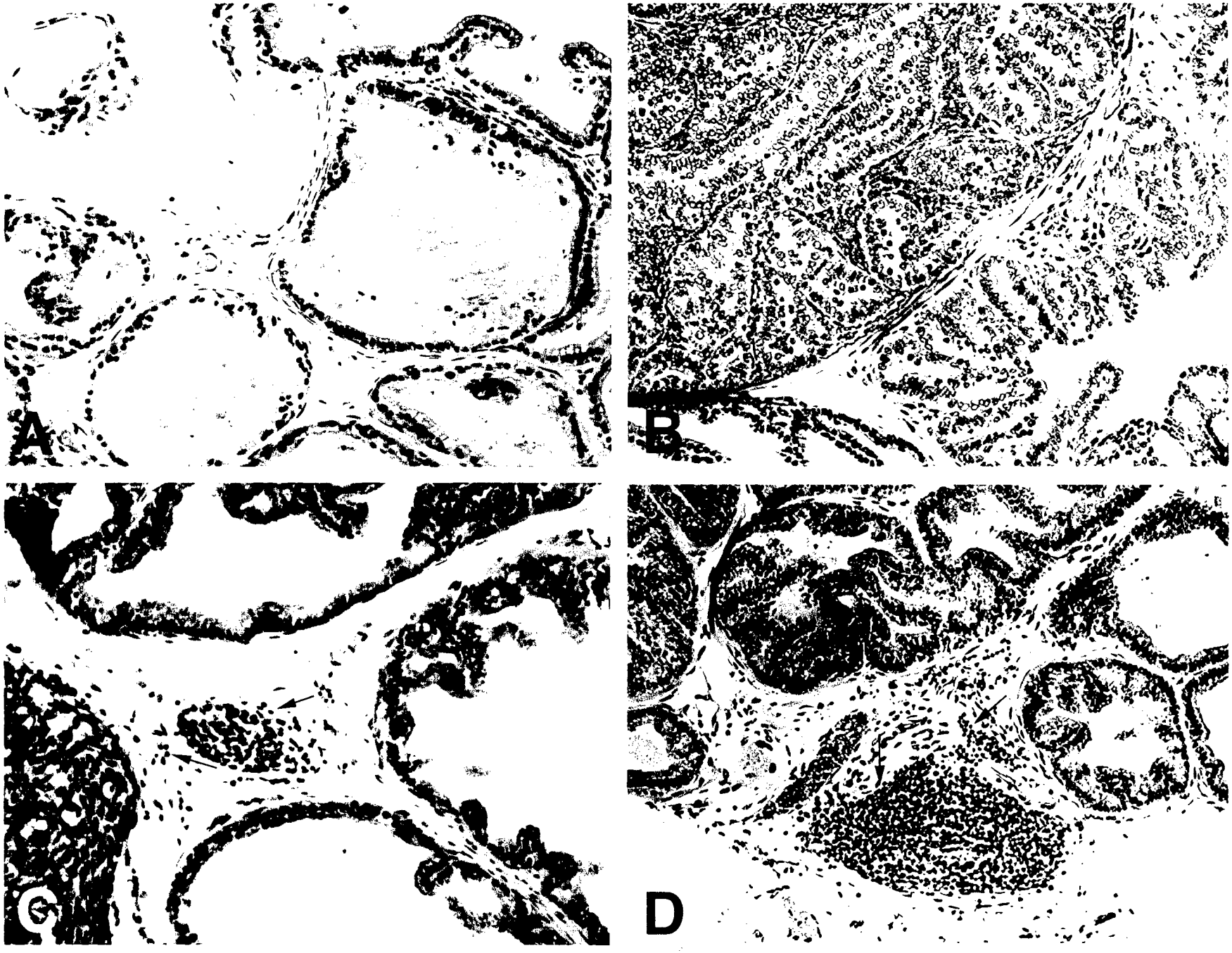
A
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